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Antigeny ze slin flebotomů a protilátková odpověď pobodaných hostitelů

Antigens in the sandfly saliva and antibody response of the bitten hosts

Dizertační práce / Ph.D. Thesis

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Praha, 2014

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supervisor

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Abstract

Leishmaniases are neglected diseases occurring mainly in developing countries of Africa, Asia and Latin America. However, these diseases are also present in Europe and North America and due to climate changes and human activities they spread to higher latitudes and altitudes. In these theses, we review the current information about the spread of leishmaniases, its vectors and reservoirs in Europe. The risk of *Leishmania* transmission is closely connected with the host-vector contact. Recently, immunoglobulin G (IgG) antibody response to sandfly saliva has been proven as a reliable marker of the host exposure. However, sandfly saliva is a complex mixture of components with different chemical and antigenic properties and it is laborious and expensive to acquire. Therefore, we have focused on preparation of major salivary antigens in the form of recombinant proteins that would be capable to replace the saliva in immunological screenings. We choose two European vectors, *Phlebotomus (P.) papatasi* and *Phlebotomus (P.) perniciosus* and identified their major salivary antigens by western blotting and mass spectrometry. We expressed these proteins in the bacterial system and test their antigenicity using ELISA and western blotting with sera of mice and dogs bitten by these sand fly species. The most promising *P. perniciosus* antigens were then used for screening the sera from various reservoirs of *Leishmania (L.) infantum* in southern Europe – dogs, hares and wild rabbits. In animals living in endemic foci of *L. infantum*, ELISA revealed high levels of specific antibodies against *P. perniciosus*. These experiments confirmed the capability of recombinant salivary proteins to replace the whole saliva. Therefore, recombinant salivary proteins of sand flies can be used as a tool for screening the exposure of host to sand fly vectors.

Abstrakt

Leishmanióza je onemocnění, které se vyskytuje především v rozvojových zemích Afriky, Asie a Latinské Ameriky a jehož význam je velmi podceňován. Zároveň je toto onemocnění přítomno také severní Ameriky a Evropy a kvůli globálnímu oteplování se šíří spolu se svými rezervoáry a přenašeči – flebotomy do vyšších nadmořských výšek a oblastí severnějších a jižnějších zeměpisných šířek. V této dizertační práci proto shrnujeme současné informace o šíření leishmaniózy a jejích přenašečů a rezervoárů a v Evropě. Riziko přenosu leishmaniózy je úzce spjato s pravděpodobností kontaktu s flebotomy a protilátková odpověď založená na imunoglobulinech typu G (IgG) proti jejich slinám byla prokázána jako vhodný nástroj pro zjištění poštípání napadených hostitelů. Sliny flebotomů ovšem mají několik nevýhod pro tuto metodu – jedná se o směs mnoha látek s různými chemickými a antigenními vlastnostmi a jejich získání přímo z flebotomů je velmi pracné a nákladně. Proto jsme se zaměřili na přípravu rekombinantních proteinů, které budou zastupovat nejsilnější slinné antigeny a nahradí tak slinné žlázy jako základ screeningových metod. Zvolili jsme dva druhy evropských flebotomů – *Phlebotomus (P.) papatasi* a *Phlebotomus (P.) perniciosus*, a identifikovali jsme nejvíce antigenní proteiny v jejich slinných žlázách, které jsme posléze exprimovali v bakteriálním expresním systému. S jejich pomocí jsme provedli průzkum sér několika druhů zvířat, která slouží jako hlavní rezervoár *Leishmania infantum* v jižní Evropě: psů, zajíců a divokých králíků a u všech těchto zvířat jsme nalezli vysokou míru kontaktu s flebotomy *P. perniciosus*. Tyto experimenty také potvrdily schopnost námi exprimovaných proteinů nahradit plně slinné žlázy jako nástroj pro zjišťování míry expozice k flebotomům.

Introduction

1. Leishmaniasis in Europe.

Leishmaniasis is a worldwide-spread parasitic disease which is transmitted by its exclusive vectors – sandflies (*Diptera: Phlebotominae*) to the definitive hosts – mammals and reptiles. Human infection (HumL) is caused by about 21 of 30 *Leishmania* species that infect mammals (http://www.cdc.gov/parasites/leishmaniasis/biology.html, Ready, 2010). It is transmitted by fewer than 150 (42 *Phlebotomus* species in the Old World and 56 *Lutzomyia* species in the New World) of the approximately 800 species of sandflies described worldwide (Maroli *et al.,* 2013). Leishmaniasis causes in mammals primary skin infection (cutaneous leishmaniasis) which sometimes resolve without treatment, but the infection can spread and produce secondary lesions in the skin (diffuse cutaneous leishmaniasis), in the mucosa (muco-cutaneous leishmaniasis) and in the spleen, liver and bone marrow (visceral leishmaniasis) which is usually fatal if untreated (Ready, 2010).

In Europe, only two *Leishmania* species have been traditionally mentioned as the causative agents of the disease – *Leishmania infantum* in the Mediterranean and *Leishmania tropica* in Greece and its neighboring countries (Desjeux, 1996). *Leishmania infantum* is causative agent of zoonosis causing both cutaneous and visceral form of the disease and its most important reservoir is domestic dog, whereas *L. tropica* cause anthroponosis with cutaneous manifestation of the disease called the „oriental sore“ (Ready, 2010). However, this traditional view on *Leishmania* distribution in Europe has changed in the past two decades and these two species are re-emerging in foci where they were considered to being extinct and they are also spreading to new foci (Antoniou *et al*., 2013). The current findings show that also new *Leishmania* species infecting humans, *L. donovani*, was introduced to Europe and new reservoir hosts of the current *Leishmania* species have been recorded (Antoniu *et al*., 2008; Maia *et al*., 2008; Mazeris *et al*., 2010; Vilas *et al*., 2012).

Similarly, a distribution of sandfly vectors in Europe has been changing recently. Sandfly species return to their old foci where their populations have been suppressed by the DDT anti-malaric vector campaigns after the World War 2 but they also spread to new foci which may be connected with global warming and changing of various demographic and landscape factors (Dereure *et al*., 2009). Also new way of transmission – by syringes of drug addicts,

has been recorded and combination of HIV spread and great number of asymptomatic host enabled a rapid jump of HumL incidence (Alvar *et al*., 1997; Desjeux & Alvar, 2003). The World Health Organization (WHO) reports that the public health impact of leishmaniasis worldwide has been grossly underestimated for many years and new outbreaks of the disease in Europe confirm seriousness of the problem also in the developed countries (www.who.int/leishmaniasis/en/; Antoniou *et al*., 2013).

1.1. Epidemiology and distribution of leishmaniases

Although leishmaniasis has the ninth largest disease burden among individual infectious diseases, it belongs to the so called "neglected diseases" (Alvar *et al*., 2012). The lack of interest about HumL can be demonstrated on the lack of data – until 2010 cases have been recorded in only 32 of the 98 countries where 350 million people are at risk (Desjeux, 2004; Alvar *et al*., 2012). In total, official case counts demonstrated more than 58,000 VL cases and 220,000 CL cases per year. Using an overall case-fatality rate of 10%, a tentative estimate of 20,000 to 40,000 leishmaniasis deaths per year was reached (Alvar *et al*., 2012). The endemic zones are located in Latin America, Africa, the Indian subcontinent, the Middle East and the Mediterranean region (Ready, 2010). The incidence of leishmaniasis is not uniformly distributed in the endemic areas: about 90% of cutaneous leishmaniasis cases occur in seven countries only (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria), whereas 90% of visceral leishmaniasis cases occur in rural and suburban areas of five countries (India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil) (Gramiccia & Gradoni, 2005, Alvar *et al*., 2012).

In Europe, HumL is present in Greece, Italy, Portugal, Malta, Cyprus, France, Spain and in former Yugoslavia (Dakic *et al*., 2009; Sisko-Kraljevic *et al*., 2013; Antoniou *et al*., 2013; NTais, *et al*., 2013). Non-autochthonous HumL also appears regularly in the non-endemic European countries (Alvar *et al*., 2008). The incidence of zoonotic VL caused by *L. infantum* in humans is relatively low in Europe (0.02–0.49/100,000 in the general population) with an average of about 700 clinical cases reported each year in southern Europe. However, the collection of incidence data of CL caused by *L. infantum* is not yet complete (Dujardin, *et al*., 2008; Alvar *et al*., 2012).

New outbreaks of VL and CL caused by *L. infantum* occured recently in Spain where incidences increased up to 56 per 100,000 (Arce *et al*., 2013). Since late 2010, an unusual

increase of human leishmaniasis cases (VL and CL) has been observed in the south-western Madrid region, mainly in Fuenlabrada (204,838 inhabitants). From July 2009 to December 2012, 542 cases of leishmaniasis were reported in the region of Madrid to the Epidemiological Surveillance Network, of which 446 (82.3%) met the outbreak case definition: 6 were identified in 2009, 97 in 2010, 196 cases in 2011 and 147 cases in 2012. Apart from Fuenlabrada there were three affected neighbouring municipalities, Leganés, Getafe, and Humanes de Madrid, of which 158 (35.3%) were VL. This is also a big difference from the previous HumL characteristic - in Madrid, over the past decade of monitoring this disease, 90% of the reported cases were visceral. In coclusion, this was the first reported outbreak of VL and CL of such magnitude in Spain (Arce *et al*., 2013).

Portugal is a hypoendemic country for human VL (20–30 cases/year) (Campino & Maia, 2010). Also some CL have been reported recently (Lopes *et al*., 2013) and three main foci were identified in eighty's decade: Trás-os-Montes and Alto Douro region, Lisbon region and Algarve region (Branco *et al*., 2013). In France, the average number of leishmaniasis autochthonous cases notified per year between 1999 and 2012 was 22.6, mainly cases of VL (84.5%). All cases were infected in the south of France and leishmaniasis incidence is 0.22 per 100,000 inhabitants in the endemic area (Lachaud *et al*., 2013).

In Italy, VL incidence has increased in humans and dogs since the 1990s, with new foci being detected within traditional boundaries of endemic transmission but also in northern regions previously regarded as non-endemic. The classical endemic zones for VL are the Tyrrhenian littoral, the southern peninsular regions and the islands (Maroli *et al*., 2002). Human VL occurred sporadically with fewer than 40 cases/year through the 1980s, but since the early 1990s the incidence has been increasing steadily; more than 200 cases were reported throughout the country in 2000 (Gradoni *et al*., 2003). Between 1990 and 2005 two-hundred-thirty human cases, which are 11% of all Italian cases, were recorded in northern continental Italy (Maroli *et al*., 2008).

In Greece, both visceral and cutaneous leishmaniasis has spread during the last 35 years, with autochthonous human cases appearing in 41 of the 54 prefectures, the majority in the Attiki prefecture (mostly in the city of Athens, which is an endemic VL area; NTais *et al*., 2013). Between 2005 and 2010, 326 autochthonous human leishmaniasis cases were reported: 297 VL and 30 CL, some CL being caused by *L. tropica* (Frank *et al*., 1993; NTais *et al*., 2013). In the Greek island of Crete, CL caused by *L. tropica* (its traditional designation is

'Chaniotico spyri') has re-emerged after 25 years (Christodoulou *et al*., 2012). Nineteen CL cases has been recorded during the years 2009-2012, 15 of them were over 60 years old. Possibly age-related changes in the immune system of these patients allowed the parasite to become activated and cause new lesions. CL has spread to all parts of Crete, with an average of five CL cases per year observed in these three years (Christodoulou *et al*., 2012).

In Cyprus, the only leishmaniasis present in the southern part of the island used to be *L. infantum* causing CanL without almost any reported human cases (Mazeris *et al*., 2010); a single infantile VL case was reported in 1987 (Minter & Eitrem, 1989). The situation is different in the northern part of the island which is not under control of the Government of the Republic of Cyprus, where an increasing number of human CL and VL, as well as CanL cases have been reported (Howard *et al*., 1992). Although the population of the two parts of Cyprus has been free to cross the green line since 2003, human leishmaniasis cases were not reported in the southern part until 2006, when three CL and two VL human cases were diagnosed (Antoniou *et al*., 2008). All of the human cases were determinated as *L. donovani* and the responsible strain - MON-37 is very closely related to the Turkish *L. donovani* MON-37. This indicates that the parasite may have been introduced to Cyprus recently, probably from mainland Turkey, where HumL is widespread. All evidence indicates that two different transmission cycles are taking place on the island, one of *L. infantum* in dogs and one of *L. donovani* in humans (Antoniu *et al*., 2008).

Leishmania infantum is endemic also in the isles of Malta. The burden of HumL is highest in children with both cutaneous and visceral manifestations. After the WW2, the eradication of stray dogs, improved sanitary conditions and urbanization have resulted in a drastic decrease in the incidence of human VL from 67/100,000 population in 1948 to 8.2/100,000 population in 1955 and down to a mean of 1.1/100,000 over the last 10 years (Pace *et al*., 2011). Children below 3 years of age suffer the majority of the visceral disease burden (Grech *et al*., 2000). The case fatality rate is extremely low with only two individuals, aged >65 years, dying from VL since 1991 (Department of Health Information, Malta. 1991–2007. Malta National Mortality Register). Rates of cutaneous disease have remained low until 1983 (mean 1.87/100,000). However, being a non-life-threatening illness and often not notified or unrecognised, the incidence of CL is confounded by underreporting (Pace *et al*., 2011).

In former Yugoslavia, VL was endemic in Macedonia, southern Serbia, the Montenegro coast, the south of Herzegovina and Dalmatia (Miscevic *et al*., 1998). During the period 1945–1955, three epidemic waves of VL were recorded in Serbia. Rare autochthonous cases were noticed in 1968 and 1969 in Nis, where *P. major, P. simici* and *P. perfiliewi* were identified according to Miscevic *et al*. (1998). According to epidemiological data, 39 VL cases were reported in Serbia and Montenegro from 1991 to 2000, with only one case being imported. In central Serbia, the incidence was 0.01/100 000 in 2007. In a retrospective epidemiological and diagnostic study of VL, which was carried out during the period 2001-2007, 22 cases of VL were diagnosed, the majority of which involved individuals who had been on holiday at the Montenegrian sea coast (Dakic *et al*., 2009). In Croatia, Human VL and canine VL have been reported in central and southern coastal and insular Dalmatia (from Split to Dubrovnik) since 1930 (Tartaglia, 1949). From 1931 to 1957, 398 human VL cases were diagnosed in this region (Tartaglia, 1957). A case of CL was firstly recorded in 1945 and 201 CL cases were recorded by 1957. After the late 1950s, the number of VL cases declined, probably again because of mass spraying with antimalarial insecticides (Tartaglia, 1957). The incidence of human leishmaniases recorded in southern Dalmatia over the past 20 years (0.4/100,000) indicates a sporadic/hypoendemic status of the diseases, similar to that of neighboring western Mediterranean countries (e.g. Italy, 0.6/100,000), and much lower than in Albania (7/100,000), where a recent VL recrudescence has been recorded (Velo *et al*., 2003). Recently, asymptomatic *L. infantum* infections in humans living in endemic and non-endemic areas of Croatia have been detected (Sisko-Kraljevic *et al*., 2013), residents of coastal areas and islands were significantly more seropositive than mainland residents and a highly endemic focus was identified in central coastal Dalmatia (seroprevalence 22.2%; Sisko-Kraljevic *et al*., 2013).

HIV infections have a significant impact on the HumL cases, especially in the asymptomatic human carriers of *L. infantum* in southern Europe (Alvar *et al*., 1997). HumL is considered to be the third most frequent opportunistic parasitic disease after toxoplasmosis and cryptosporidiosis (Desjeux & Alvar, 2003). Cases of the co-infection have been reported from 35 countries around the world but most of them have been recorded in south–western Europe. By early 2001, the cumulative number of cases recorded by the WHO was 1,911, of which 57% were from Spain, the rest of the cases were reported from France, Italy and Portugal (Desjeux & Alvar, 2003) and additional 241 new cases of primary infections from

Spain (95 cases), Portugal (64 cases), Italy (52 cases), and France (30 cases) were reported to WHO during the period 2001-2006; some persistent cases were also reported. Other countries, such as Switzerland, Germany, United Kingdom, and Greece, reported sporadic imported cases (Alvar *et al*., 2008). Highly active anti-retroviral therapy (HAART) treatment has reduced the incidence of co-infections with *Leishmania* and HIV by preventing an asymptomatic infection with *L. infantum* from becoming symptomatic, but unfortunately it is not good at preventing visceral leishmaniasis relapses**.** The benefits of treatment are not as clear-cut as they are for other opportunistic diseases (Lopez-Velez, 2003).

1.2. Vectors

Two sandfly genera are involved in transmission of human *Leishmania*: *Lutzomyia* in the New World and *Phlebotomus* in the Old World. In Europe, seven proven *Leishmania* vector species are present - *P. ariasi, P. perniciosus, P. perfiliewi, P. neglectus, P. tobbi, P. papatasi* and *P. sergenti*, and besides *P. sergenti* and *P. papatasi,* all of them are vectors of *L. infantum* (Killick-Kendrick, 1999; Ready, 2010). These two sandfly species are specific vectors of two different *Leishmania* species – *P. sergenti* transmits *L. tropica* and *P. papatasi is a specific vector of L. major.*

The specificity of the vector is determined by the composition of midgut surface molecules which enable *Leishmania* attachment to the midgut surface and thus prevent them from being defecated with the digested blood (Volf & Myšková 2007). The European proven vectors of *L. infantum* (all of them belonging to the subgenus *Larroussius*), in contrary, allow different *Leishmania* spp. to mature in their midgut, thus falling into a category of permissive vectors. This fact is important for the epidemiology of leishmaniasis – new *Leishmania* species can be introduced into the present vectorial cycles – this explains some unexpected findings from the field such as the circulation of *L. tropica* through the permissive vector *P. arabicus* (Jacobson *et al.*, 2003; Svobodova *et al.*, 2006) and the introduction of *L. infantum* (syn. *L. chagasi*) from the Mediterranean to Latin America, where it adapted to the local permissive sandfly *L. longipalpis* (Mauricio *et al.*, 2000). The susceptibility of many sandfly species has not been identified even at the European sandflies – for example *P. mascittii* is possible but unproven *Leishmania* vector and some species still have questionable taxonomic status – e.g. *P. similis* and *P. syriacus* (Ready, 2010). *Phlebotomus* species occurring in Europe belong to four subgenera: *Phlebotomus,*

Paraphlebotomus, Larroussius and *Transphlebotomus*. The most widespread subgenus in Europe is *Larroussius* which includes vectors of *L. infantum* and *L. donovani*. The *Larroussius* species with the widest distribution, *P. perniciosus*, is present in both the southern and northern parts of the Mediterranean, from Morocco and Portugal in the west to Italy in the east and Germany in the north (Killick-Kendrick, 1999). The concurrent distribution of all the HumL vectors in Europe is described in Table 2 in the review article of Ready, 2010. However, this distribution has been rapidly changing in the past few decades, so it is not invariant – sandfly species are returning to their old foci from where they were banished together with mosquitos due to the DDT anti-malaria campaigns and they even spread to new foci probably in connection with the global warming (Antoniou *et al*., 2013).

In Portugal, *P. perniciosus* and *P. ariasi* are the proven vectors of *L. infantum* (Branco *et al*., 2013). From June to November 2010, 275 biotopes in Torres Novas municipality (Santarém District in central Portugal), were surveyed with CDC light-traps - a total of 1262 sandflies were captured and identified, and four species were detected: *P. perniciosus* (73.69%), *P. ariasi* (8.16%), *P. sergenti* (6.58%) and *Sergentomyia minuta* (11.57%). In 71.4% localities at least one *L. infantum* proven vector species was present. *Leishmania infantum* infection rate was 4% for *P. ariasi* and 0.48% for the total of *Larroussius* females (Branco *et al*., 2013). Recently, also genetic hybrids between two very divergent *Leishmania* species: *L. infantum* and *L. major* were isolated in Portugal from immunocompromised patients (Ravel *et al*., 2006).

In Spain, a sandfly surveillance system recently implemented in the region of Madrid, showed increase in the density of *P. perniciosus* in the epidemic area (16 sandflies/m^2 in 2008, 30 sandflies/m^2 in 2010 and 50 sandflies/m^2 in 2012; Iriso *et al*., 2013). This finding correlates with the leishmaniasis outbreak as it was described in the previous chapter. Another study (Arce *et al*., 2013) came with even higher results from the area of the leismaniasis outbreak - in 2012, the mean density was very high, reaching 143.8 sandflies/m^2, detecting predominance of *P. perniciosus* (66.1%), the principal vector of *Leishmania* in the region. An extension of presence of this vector both in latitude and altitude has also been observed. Recent changes in the environment - large road improvement works in some towns of the outbreak and warm autumns, may have contributed to the high density (Arce *et al*., 2013). Moreover, molecular detection of *L. infantum* in wild-caught *P. perniciosus* (n=135) showed a very high prevalence of infection

(58.5%) providing evidence of the high transmission events that are taking place in the focus (Jiménez *et al*., 2013). Similar increase of sandfly vectors (*P. ariasi* and *P. perniciosus*) has been postulated in southern France because of recent growth of CanL cases in the foothill villages without any major changes in land use (Dereure *et al*., 2009).

In northern Italy, an increase in the density and geographical expansion of the *Leishmania* vectors *P. perniciosus* and *P. neglectus* was observed in 2003 and 2004 compared with the situation described in the 1960s and 1970s (Biocca *et al*., 1977); this enabled the establishment and transmission of the parasite in the northern part of the country previously regarded as non-endemic (Maroli *et al*., 2008). *Phlebotomus perniciosus*, already detected at low and medium density in a few spots in four regions, has now been found at high densities in most collecting sites in hilly and low mountain ranges of six regions. *Phlebotomus neglectus*, which was never reconfirmed in northern Italy after its first identification in 1917 (Tonnoir 1921), was re-collected since 1995 in several pre-Alpine sites of five regions and abounded in some of them (Maroli *et al*., 2002, 2006; Ferroglio *et al*., 2005).

In Greece, a recent faunistic study of phlebotomine sandflies was carried out on the mainland and on four Greek islands - Crete, Samos, Rhodos and Cephalonia between 1999 and 2004 (Ivovic *et al*., 2007). A total of 8688 sandflies were collected and a total of 10 species were identified. Of these, the species with the widest distribution were *P. perfiliewi* Parrot, *P. tobbi* and *Sergentomyia minuta* Rondani, then *P. neglectus* and *P. simici* Nitzulescu. The species *P. neglectus* Tonnoir and *P. perfiliewi* Parrot are epidemiologically the most important vectors of leishmaniases and sandfly fever in Greece. They were shown to be present in the main endemic foci of the country. Despite the fact that 80% of the terrain of Greece is mountainous, mainly the central part dividing the country into two, the same *Phlebotomus* spp. were found in the eastern and the western parts (Ivovic *et al*., 2007). In Crete alone, an even bigger study was carried out in various months during the whole sandfly season (May to October), from 2001 to 2010 (Christodoulou *et al*., 2012). A total of 14,563 sandflies were trapped in nine areas during the summer months of the years 2001–2010. They belonged to nine different species of *Phlebotomus* and one of *Sergentomyia. Phlebotomus neglectus* (proven vector of VL) and *P. similis* (a suspected vector of *L. tropica)* were found in all sampling sites in the main island (Christodoulou *et al*., 2012).

In Cyprus, a total of 1,716 sandflies, comprising 10 species, were collected in 2006 (Mazeris *et al*., 2010). *Phlebotomus papatasi* was found in almost all areas studied (18/20), *P. tobbi* 14, *P. galilaeus* in 7, *P. sergenti* in 4, while *P. alexandri*, *P. mascittii* and *P. economidesi* only in 1 of 20 areas studied. However, further studies should investigate the capacity of other species to transmit *L. donovani* in Cyprus, such as *P. galilaeus* (Mazeris *et al*., 2010).

In Germany, two sandfly species were detected, *P. perniciosus* was found in association with an autochthonous case of canine leishmaniasis near Kaiserslautern and 237 specimens of *P.* (*Transphlebotomus*) *mascittii* were caught in 17 different locations in Baden-Wuerttemberg and Rhineland-Palatinate. The northernmost finding in Germany (and Europe) was a female of *P. mascittii* caught in Giessen in the German state of Hesse during an entomological survey in July 2013 (Melaun *et al.*, 2014). *Phlebotomus mascittii* has not yet been confirmed as a vector of leishmaniasis, but its competence is strongly suspected. In addition to the detection of the vector, since 1991, there have been 11 cases of leishmaniasis in Germany in which an autochthonous origin was confirmed or which were highly likely to have been of indigenous origin. Data from the German meteorological service indicate that Germany currently has a Mediterranean climate, with an annual average temperature of 10°C being reached or exceeded in several regions (areas along the Rhine rift up to Frankfurt and around Cologne/Bonn). This type of climate is also appropriate for the living conditions of sandflies. Therefore, it is assumed that sandflies have a greater geographical distribution in Germany than the first studies suggested, being mainly restricted to the southern region of Baden-Wuerttemberg. *P. mascittii* has already been found in neighbouring countries: in western and southern Switzerland in 1912 and 1931, respectively (Galli-Valerio, 1912; Vogel, 1931), in France in 1950 close to the German border in Strasbourg (Callot 1950), and recently in 2001 in Sainte-Cécile (Florenville) in Belgium (Depaquit *et al*., 2005).

Miscevic *et al*. (1998) have reviewed sandfly studies carried out in the former Yugoslavia during the period 1969–1990. Collections performed in Makarska, Split and Omiš, documented the presence of five species: *P. papatasi, P. neglectus, P. perfiliewi, P. tobbi and S. minuta.* An entomological survey was carried out in 2002-2004 in collecting sites of three counties of southern Dalmatia: Sibenik-Knin, Split-Dalmatia and Dubrovnik-Neretva, with about 700,000 population (Bosnic *et al*., 2006). All sites monitored were positive for

sandflies. An over total of 2917 phlebotomine specimens were collected during the 3-year survey. Five species were identified, four of which belonging to the *Phlebotomus* genus (three being proven *L. infantum* vectors) and one to the *Sergentomyia* genus. *Phlebotomus tobbi* was the most widespread species, being recorded in all sites monitored except in Komolac (Dubrovnik-Neretva county). *P. neglectus* was apparently absent only in Slivno (Šibenik-Knin county). As for the other species, *P. perfiliewi* was caught in four sites only, and *S. minuta* was most abundant in Zastražišće (island of Hvar), where also three specimens of *P. mascittii* were caught (this being the first record of this species in the former Yugoslavia; Bosnic *et al*., 2006).

Other important sandfly species occurring in Europe belong to genera *Phlebotomus* (a single species *P. papatasi)* and *Paraphlebotomus* (two species, *P. sergenti* and *P. similis*). The geographical range of *P. sergenti* extends to Spain, Portugal and Italy (Sicily), where genetic competence of local sandfly populations for anthroponotic *L. tropica* transmission has been suggested (Depaquit *et al*., 2002). In Greece all of the previous continental or insular records of *P. sergenti* are actually *P. similis* which was found in 11 of 19 prefectures studied (NTais *et al*., 2014). This species is believed to be the potential vector of *L. tropica* in Crete, which is a fact that needs further investigation. It is present also in Ionian Islands and Crete where CL is widespread but also in areas on the coast of the Aegean Sea where CL cases have never been reported (Christodoulou *et al*., 2012). The same is true for Malta (Gozo), where *P. similis* is present, not *P. sergenti*, as reported by Leger *et al*. (1991). This was proven by comparing of the internal transcribed spacer 2 (ITS2) sequences (Depaquit *et al*., 2002). *Phlebotomus sergenti* was frequently reported also in eastern Serbia and Macedonia (Zivkovic, 1980) but this could be probably also misidentification with *P. similis*).

Phlebotomus papatasi, the vector of *L. major* causing zoonotic cutaneous leishmaniasis which is found in northern Africa and the Middle East, is present also in Albania, Cyprus, Greece, Italy, Portugal, Spain and countries of the former Yugoslavia (Bosnic *et al*., 2006; Depaquit *et al*., 2008; NTais *et al*., 2014).

1.3. Reservoirs of *Leishmania infantum*

The most spread *Leishmania* species in Europe – *L. infantum,* cause a zoonotic disease. For this reason monitoring and control of the reservoir animals are essential. The primary reservoir animal is domestic dog which suffers both CL and VL - the visceral form of

the disease is lethal for the dog without treatment. CanL has been spreading in Europe during the past few decades together with the spread of HumL and its vectors and also new reservoir animals have emerged (Antoniou *et al.*, 2013). Wild canids like red foxes, wolves and badgers are hosts of *L. infantum* that are involved in the sylvatic cycle which contributes to the sandfly infectivity (Quinell *et al.*, 2009). Although their populations are apparently healthy, the prevalence of infection (in the red fox can be as high as 40–75% in Mediterranean countries; Criado-Fornelio *et al.*, 2000; Dipineto *et al.*, 2007) indicates the existence of natural infection (Quinell *et al.*, 2009). This finding is also supported by the evolutionary history of *L. infantum*: since *L. infantum* diverged from *L. donovani* around 1 million years ago, well before the domestication of dogs around 15 000 years ago. This ancestral reservoir is often assumed to be a wild canid, though rodents have also been suggested (Ashford, 2000).

Importantly, existence of noncanid reservoir hosts has been demonstrated in the past decade – most important are cats and hares (Antoniou *et al.*, 2013). Also horses and other domestic equines suffer occasionally of single or multiple cutaneous lesions and they probably represent an incidental host of the disease (Gramiccia, 2011). Besides that, another wild carnivores and rodent have been found infected by *Leishmania: Mus spretus* (Algerian mouse), *Apodemus sylvaticus* (European wood mouse), *Rattus rattus* (black rat), *Rattus norvegicus* (brown rat), *Martes martes* (European pine marten), *Mustela nivalis* (weasel), *Geneta geneta* (common genet) (Antoniou *et al.*, 2013). Most rodent studies have concentrated on the black rat (*Rattus rattus*), for which reported parasite prevalence is only 1–2% (Bettini *et al.*, 1978, 1980; Pozio *et al.*, 1981), but only limited PCR studies have been performed.

Some recent studies have shown a relatively high frequency of sexual and congenital transmission in dogs. Sexual transmission was demonstrated in 58% (7/12) of uninfected bitches mated to multiple infected dogs (Silva *et al.,* 2009), and congenital transmission to 26% (8/31) of puppies born to 7 bitches in Italy (Masucci *et al.,* 2003). In contrast, none of 56 puppies born to 18 infected bitches in a Brazilian study were infected by congenital transmission (Andrade *et al.,* 2002). There is no evidence that sexual and congenital routes can sustain transmission in the absence of sandflies but it can explain several autochthonous cases of canine leishmaniases in areas where sandflies are absent or rare (Naucke & Lorentz, 2012).

Establishment of several new *L. infantum* foci was recently demonstrated in Spain. In Alpujarras region of southeastern Spain, seroprevalence of CanL has progressively increased over 22 years (1984–2006), climbing from 9.2% in 1984 to 15.4% in 1991 and finally to 20.1% in 2006 (Martin-Sanchez *et al.*, 2009). Interestingly, during the outbreak of HumL in Spain in Madrid in the years 2009-2012 dogs were not the main reservoir (Arce *et al.*, 2013). Data derived from analysis of 2,070 dogs studied during 2011 and 2012 revealed a low canine prevalence of 1.64% in the affected municipalities (Vilas *et al.*, 2012). Therefore, other potential reservoirs were tested and *Leishmania* DNA was detected in spleen and/or skin of 29% hares, 1.5% rabbits and 7.3% cats (Vilas *et al.*, 2012). In addition, xenodiagnosis studies have demonstrated for the first time that apparently healthy Iberian hares (*Lepus granatensis*), seropositive to *L. infantum*, were able to infect *P. perniciosus* (Molina *et al.*, 2012). Recent observations on *P. perniciosus* blood meal preferences (Jiménez *et al.*, 2013) and the high population of hares present in the newly constructed periurban green park suggest that hares are maintaining a high sandfly population in the area and they are playing an active role as reservoirs in a sylvatic transmission cycle linked to the urban periphery independent of the usual domestic one (Molina *et al.*, 2012; Jiménez *et al.*, 2013). In addition, a recent study based on molecular detection of the parasite in spleen of hares captured in different areas of Spain, showed a high prevalence of infection (42%) in *L. granatensis* and *L. europaeus* (Ruíz-Fons *et al.*, 2013).

Cats were also diagnosed for leishmaniasis in southern Spain (Martin-Sanchez *et al.*, 2007). In 2011, 183 cats were tested with the resulting seropositivity value of 60.0%. Around 25.7% of the cats studied were parasitemic and some of them remained positive for months. Combining both data, 70.6% of the feline population was, or could be, infected (Martin-Sanchez *et al.*, 2007). In the wild canids in Spain, DNA of the parasite was detected in spleen or blood samples from 35 (16.12%) analyzed wild carnivores, including 8 of 39 (20.5%) wolves (*Canis lupus*), 23 of 162 (14.1%) red foxes (*Vulpes vulpes*), 2 of 7 (28.6%) Egyptian mongooses (*Herpestes ichneumon*), 1 of 4 genets (*Geneta geneta*), and 1 of 4 Iberian lynxes (*Lynx pardinus*; Sobrino *et al.*, 2008).

In the Algarve region, which is an international tourist destination in the southern Portugal, an overall CanL seroprevalence of 16.06% was found; the seroprevalence was 3.88% in dogs housed in kennels and 40.63% in dogs that attended veterinary clinics (Maia *et al.*, 2013). In the second CanL foci - in Torres Novas municipality, Santarem District, canine

seroprevalence was 7.93% (Branco *et al.*, 2012). In addition, 23 adult stray cats from endemic regions in Portugal (all of them asymptomatic) were surveyed by clinical examination, and peripheral blood samples for serological and molecular analysis were collected (Maia *et al.*, 2008); *Leishmania* DNA was detected in blood of 7 cats (30.4%). Authors therefore suggest that cats may act as a habitual reservoir host of *L. infantum* infection in these endemic areas (Maia *et al.*, 2008). In the Lisbon metropolitan area, another endemic focus of zoonotic leishmaniasis, *L. infantum* DNA was detected in peripheral blood of 28 out of 138 cats (20.3%) but positive serology was observed only in one cat out of 76 (Maia *et al.*, 2010). In the same geographic region and time period, the indirect immunofluorescent test revealed 20.4% (31/152) of dogs with antibodies and PCR detected *Leishmania* DNA in 34.9% (53/152) animals. These results correlate with the preliminary serological investigation which have shown that *Leishmania*-infected cats often developed a low level of antibodies or remain seronegative (Poli *et al.*, 2002; Martin-Sanchez *et al.*, 2007). There are two possible explanations: the absence of antibodies could be related with the fact that exposed/infected cats do not suffer from disequilibrium of the immune status leading to over-production of antibodies as it occurs in dogs, or conventional serology does not seem to be reliable enough in cats (Maia & Campino, 2011).

In southern France, CanL seems to spread as well (Dereure *et al.*, 2009). In 1994, the mean seroprevalence of 336 dogs from villages in south Pyrenees was 11.67% in the valley villages and 1.43% in the foothill villages. Interestingly, in 2007, (216 canine samples) the ratio switched – seroprevalence in the valley villages was only 2.72% and 11.32% in the foothills villages. The decrease of seroprevalence in the valley villages is probably related to use of deltamethrin collars during the transmission season. On the other hand, the increase in the foothill villages supports the climate change theory, since there was an increase of about 1°C in the mean annual temperature (Dereure *et al.*, 2009).

Wild carnivores may also to contribute to *L. infantum* circulation in France. Red foxes in the Var area, southeastern France, were found infected in the years 2006 to 2012, when a longitudinal epidemiologic survey of foxes using quantitative PCR was conducted. Among 92 red foxes screened, prevalence of *L. infantum* infection was 9% (Davoust *et al.*, 2014).

In Italy, the classical endemic zones for CanL are the Tyrrhenian littoral, the southern peninsular regions and the islands, where *P. perniciosus* acts as the main vector and seroprevalence rates in dogs may exceed 40% (Maroli *et al.,* 2001). But during the period

from 2002 to 2009, the northward spread of CanL was monitored in northern Italy with mean seroprevalences increasing from 1.8 to 4.7% (Maroli *et al.*, 2008). The role of wild carnivores and cats in Italy might be even more important than in above mentioned countries (Spain, Portugal and France). In red foxes, very high value of 40% PCR positivity was detected - fifty samples were collected from May 2004 to May 2005 in southern Italy (Dipineto *et al.*, 2007). After the occurrence of first clinical case of feline leishmaniasis (FL) in a FIV+ cat (Feline Immunodeficiency Virus similar to HIV in humans) in Sicily in 1997, a retrospective serologic trial (IFAT) was performed in 57 FIV+ and 36 FIV- cats living in the eastern part of the island (Pennisi, 2002). Fifty-five samples (59%) had high anti-*Leishmania* seroprevalence. FIV- cats had a lower prevalence (42%), while FIV+ cats had a prevalence of 70% and higher antibody titres (Pennisi, 2002).

In Greece, 5,772 dogs were examined in the years 2005–2010 (NTais *et al.*, 2013). This sample was shown to be statistically representative in terms of geographical spread and canine characteristics. Average seroprevalence was 22.09% (ranging from 6.5% in western Macedonia, to 50.2% in the island of Corfu) and seropositive dogs were found in nearly all prefectures from which dog samples were available (41/43 prefectures) indicating the spread of the disease in the entire country. Ninety-five of the seropositive dogs (0.02%) were without any obvious leishmaniasis signs and the parasite was isolated from 14 such animals (NTais *et al.*, 2013). In addition, blood of 284 stray cats was examined by serological methods in northern Greece (Thessaloniki). The prevalence was 3.87% (11 cats), this was lower than in dogs coming from the same area, based on previous studies (Diakou *et al.*, 2009).

From 1951 to 1983, only few CanL cases were reported in Crete (Chaniotis 1994). Since then seroepidemiological studies in dogs, during the last 25 years, showed that the number of seropositive animals increases with time (Antoniou *et al.*, 2009): from 0.27% in 1990 (Data of the Greek Ministry of Agriculture) to 2.9% in 1994 and 19.8% in 2009. This may be explained by the fact that dogs are brought into the island from mainland, especially from Attica, where leishmaniasis is endemic. The yearly increase in the number of seropositive dogs continues (Antoniou *et al.*, 2009) and has been followed by the re-emergence and the yearly increase of human VL but also CL cases.

In Cyprus almost complete eradication of CanL was achieved by the DDT antimalarial campaign and the anti-echinococcosis dog culling campaign of 1970–1975 (Mazeris *et al.*, 2010). Then however, sandfly populations increased and the number of dogs too; as a

consequence, CanL reemerged, and new cases were recorded again in coastal areas in 1996. In contrast to HumL in Cyprus, which is caused by *L. donovani*, CanL is caused by *L. infantum* (Leger *et al.*, 2000). Currently, CanL is widespread in the island, and seroprevalence had an almost 9-fold increase in the last 10 years - overall average increased from 1.7% 7 to 15-20% (3000 canine sera tested; Mazeris *et al.*, 2010).

In Dalmatia (Croatia), starting from 1931 (Tartaglia, 1937), cases were reported from both stray and domestic dogs in several territories of the region (Zivicnjak *et al.*, 2005). Infection prevalences determined by parasitological and/or serological techniques varied from nil (e.g. in some inland villages of the Split-Dalmatia county located at high altitudes) to 42.8%, detected in dogs from the municipality of Zagvozd, in the same county. A recent cross-sectional serological survey on 306 apparently healthy dogs from Split and nearby villages revealed a mean prevalence of 15% (Zivicnjak *et al.*, 2005). Recently, the attention was paid also to the role of wild canines in this area. In the south Dalmatia was reported for the first time a gray wolf (*Canis lupus*), which was found in November 2003 with *L. infantum* lesions typical for visceral leshmaniosis commonly described in dogs (Beck *et al.*, 2008).

In Hungary, first report of autochthonous CanL infection was announced in 2007 – prevalence of dogs in a kennel in Tolna province was 30% (6/20 dogs; Tanczos *et al.*, 2012). Similarly, in Germany, few leishmaniasis cases have emerged since 1991 and four of these cases were published: one dog each in Landsberg/Lech (Bavaria) and near Cologne (Gothe 1991), one infant (Bogdan *et al.*, 2001) and one horse near Augsburg (Koehler *et al.*, 2002). Most of the cases occurred in regions where sandflies have been found or where the climate is appropriate for their living conditions (Naucke *et al.*, 2008).

2. Sandfly saliva

The saliva that spit the blood sucking insects and mites into the feeding lesion helps them to obtain a blood meal and therefore it has two main functions – 1) to inhibit haemostasis and 2) prevent the development of inflammation and an immune response (Titus *et al.*, 2006). These functions are essential not only for the vector but also for the pathogen the vector transmits as they enable the pathogen to spread in the host and fight the immune system (Rohousova & Volf, 2006).

As no fully functional commercial vaccine against human protozoan infections has been developed yet, development of a vaccine against the vectors antigens including salivary antigens is very important and the current results are optimistic (Bethony *et al.*, 2011). Another application of the salivary antigens in epidemiology of vector-borne diseases is using them as the markers of exposure to vector bites and diagnose consequently the risk of pathogen transmission (Andrade & Teixeira, 2012).

2.1. Major salivary components

The sandfly sialome (trancriptome of salivary glands) has been already mapped in 12 species belonging to 7 subgenera (Table 1 and 2).

Haemostasis is a complex mechanism that prevents blood loss from a damaged vessel. It consists of platelet aggregation, coagulation cascade and vasoconstriction; sandfly saliva affects all of these three functions. The most important triggering molecules for haemostasis are ATP and ADP. They are produced primary by the damaged cells and ADP secondary by the activated platelets, and lead to platelet aggregation, vasoconstriction and also activate leukocytes (Key *et al.*, 2009). Three enzymes degrade these molecules and their products: apyrase (sandfly apyrases belong to the *Cimex* apyrase family; Valenzuela *et al*, 2001b) that hydrolyzes ATP and ADP to AMP, and is present in all sandfly species tested (see table 2), 5´nucleotidase hydrolyzing AMP to adenosine (Ribeiro *et al.*, 1999) and an adenosine deaminase that further hydrolyzes adenosine to inosine (Charlab *et al.*, 2000). Adenosine is inherently present in the saliva of some Old World *Phlebotomus* sandflies because it has antiplatelet and vasodilatory effect (Ribeiro *et al.*, 1999). The most potent vasodilatory activity described in *Lutzomyia* species is maxadilan of *L. longipalpis* (Ribeiro & Modi, 2001).

Table 1. List of sandfly species with sequenced sialomes

Genus and subgenus	Species	*Leishmania* transmitted	Authors
Phlebotomus (Adlerius)	*arabicus*	*L. tropica*	Hostomska *et al.*, 2009
Phlebotomus (Euphlebotomus)	*argentipes*	*L. donovani*	Anderson *et al.*, 2006; Martin-Martin *et al.*, 2013a
Phlebotomus (Larroussius)	*ariasi*	*L. infantum*	Oliveira *et al.*, 2006
Phlebotomus (Larroussius)	*orientalis*	*L. donovani*	Vlkova *et al.*, 2014
Phlebotomus (Larroussius)	*perniciosus*	*L. infantum*	Anderson *et al.*, 2006; Martin-Martin *et al.*, 2013b
Phlebotomus (Larroussius)	*tobbi*	*L. infantum*	Rohousova *et al.*, 2012
Phlebotomus (Paraphlebotomus)	*sergenti*	*L. tropica*	Rohousova *et al.*, 2012
Phlebotomus (Phlebotomus)	*duboscqi*	*L. major*	Kato *et al.*, 2006
Phlebotomus (Phlebotomus)	*papatasi*	*L. major*	Valenzuela *et al.,* 2001a; Abdeladhim *et al.*, 2012
Lutzomyia (Helcocyrtomyia)	*ayacuchensis*	*L. mexicana, L. peruviana*	Kato *et al.*, 2013
Lutzomyia (Lutzomyia)	*longipalpis*	*L. chagasi*	Valenzuela *et al.*, 2004
Lutzomyia (Nyssomyia)	*intermedia*	*L. braziliens*	de Moura *et al.*, 2013

As mentioned above, two very different components causing vasodilatation are present in sandfly saliva – adenosine and 5'AMP in *Phlebotomus* spp. (Ribeiro & Modi, 2001) and maxadilan, which is a specific pituitary adenylate cyclase activating peptide type I receptor agonist, in *Lutzomyia* spp. (Moro & Lerner, 1997). Maxadilan also influences various types of vertebrate immune responses including splenocyte proliferation (Qureshi *et al.*, 1996) and inhibition of Th1 cytokine production (IL-12, TNF-α and also NO; Soares *et al.* 1998; Rogers & Titus 2003; Wheat *et al.*, 2008) cytokines together with stimulation of Th2 cytokine production (IL-6, IL-10, and TGF-β; Bozza *et al.*, 1998; Soares *et al.* 1998; Rogers & Titus 2003). Interestingly, these effects are connected with the vasodilatory activity – recombinant maxadilan without this activity did not affect the cytokine production (Rogers & Titus 2003).

During bigger injuries the coagulation cascade is also important. The salivary protein lufaxin (*L. longipalpis* Factor Xa inhibitor) which is present in most of the sandfly species saliva, affects Factor Xa, a key player at several stages of the coagulation system (Collin *et al.*, 2012).

Two enzymes involved directly in blood obtaining process are hyaluronidase and endonuclease. Hyaluronidase degrades hyaluronic acid (HA) and other glycosaminoglycan constituents present in the vertebrate extracellular matrix and therefore it helps blood draining and spreading of the other salivary factors (Stern & Jedrzejas, 2006). Hyaluronidase also inhibits immune responses because HA fragments affect DC maturation, T-cell proliferation, cytokine, and chemokine synthesis by lymphocytes and macrophages (Mummert, 2005). It is also a well-known allergen occurring in venom of many insect species (Muller, 2011). Endonuclease which is present in some sandfly species saliva probably reduces viscosity of the blood pool during feeding and liberates nucleosides (Sansom *et al.*, 2008). Some other sandfly salivary enzymes are important for digestion of the sugar meal – pyrophosphatase-like protein, amylase and trehalase (Jacobson & Schlein, 2001, Hostomska *et al.*, 2008). In *P. argentipes* saliva lipase was also detected (Anderson *et al.*, 2006).

Yellow-related proteins are common in insects and have many different functions – they play role in melanotic encapsulation of parasites in the hemocoel (Li *et al.*, 1994) and dopachrome-converting enzyme shares homology with *Drosophila melanogaster* yellow proteins (Johnson *et al.*, 2001). The recombinant yellow-related proteins from *L. longipalpis* saliva were proved to act as high affinity binders of prohemostatic and proinflammatory biogenic amines such as serotonin, catecholamines and histamine (Xu *et al.*, 2011).

D7-related and PpSP15-like proteins belong to the family of odorant-binding proteins which is composed of pheromone-binding proteins (PBP) and general-odorant-binding proteins (GOBP; Zhang *et al.*, 2014). These proteins were recently detected also in *L. longipalpis* pheromone glands (Gonzalez-Caballero *et al.*, 2013). Function of sandfly PpSP15-like and D7-related proteins remains unknown but e.g. anopheline (not sandfly) D7 proteins (Hamadarin) allow binding of biogenic amines and components of the contact activation system of coagulation (Factor XII and prekallikrein) leading to bradykinin release (Calvo *et al.*, 2006; Isawa *et al.*, 2007).

Antigen 5-related proteins belong to the CAP family of proteins which is composed of Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins (Megraw *et*

al., 1998). Ag5r proteins were described in the venom of Hymenoptera (Lu *et al.*, 1993) and they were also found in salivary glands of various bloodsucking insects like Ceratopogonidae (Campbell *et al.*, 2005) or Tabanids (Ma *et al.*, 2011). The exact function of Ag5r in sandflies is still unknown.

Several salivary proteins of sandflies contain C-type lectin or C-type lectin-like domain (Valenzuela *et al.*, 2004; de Moura *et al.*, 2013; Kato *et al.*, 2013). This putative domain may function as a $Ca2^{+}$-dependent carbohydrate-binding pocket involved in extracellular matrix organization, anticoagulation, pathogen recognition and cell-to-cell interactions (Weis *et al.*, 1998).

The PpSP32-like proteins and ParSP25-like proteins have no homologs in accessible databases and their function remains unknown but they are abundant in many different sandfly species saliva (see Table 2). Some ParSP25-like proteins were demonstrated to be immunogenic (Martin-Martin *et al.*, 2012; Hostomska *et al.*, 2008) but PpSP32-like protein from *L. longipalpis* did not elicit either specific humoral or cellular response (Xu *et al.*, 2008). Also many other proteins and peptides are present in sandfly saliva but their function has not been discovered yet.

Table 2. Proteins in sandfly saliva. C – protein sequence included in cDNA library, m – protein identified by mass spectrometry. Sandfly subgenera*: Adl – Adlerius, Eup – Euphlebotomus, Lar – Larroussius, Par –Paraphlebotomus, Phl – Phlebotomus, Hel – Helcocyrtomyia, Lut – Lutzomyia, Nys – Nyssomia*. Sandfly species: *Ara - P. arabicus, Arg - P. argentipes, Ari - P. ariasi, Ori - P. orientalis, Per - P. perniciosus, Tob - P. tobbi, Ser - P. sergenti, Dub - P. duboscqi, Pap - P. papatasi, Aya - L. ayacuchensis, Lon - L. longipalpis, Int - L. intermedia*

	Adl	***Eup***	***Lar***			***Par***		***Phl***		***Hel***	***Lut***	***Nys***
Protein family	***Ara***	***Arg***	***Ari***	***Ori***	***Per***	***Tob***	***Ser***	***Dub***	***Pap***	***Aya***	***Lon***	***Int***
5´nucleotidases											c, m	c, m
adenosine deaminase								c, m			c, m	c, m
amylases	c, m			c					c		c	
Ag 5-related proteins	c, m	c, m	c	c, m	c, m	c, m	c, m	c, m	c	c	c	c, m
apyrases	c, m	c, m	c	c, m	c, m	c, m	c, m	c, m	c, m	c	c, m	
C-type lectins										c	c, m	c, m
D7-related proteins	c, m	c, m	c	c, m	c, m	c, m	c, m	c, m	c, m	c	c, m	c, m
endonucleases	c	c		c	c							c, m
hyaluronidases	c			c		c					c	c, m
lipases		c										
lufaxins	c, m	c, m	c	c	c, m	c, m		c, m	c	c	c	c, m
maxadilan											c, m	c
ParSP25-like proteins	c, m		c	c	c	c, m						
phospholipases	c		c	c	c							
PpSP15-like proteins	c, m	c, m	c	c, m		c, m	c, m	c, m	c, m	c	c, m	c, m
PpSP32-like proteins	c, m	c	c	c	c, m	c, m	c	c, m	c, m	c	c	
pyrophosphatases	c	c		c				c				
RGD-like peptides										c	c	
trehalases	c											
yellow-related proteins	c, m	c, m	c	c, m	c, m	c, m	c, m	c, m	c, m	c	c, m	c, m

2.2. Transmission blocking vaccine based on sandfly salivary components

Immunisation with sandfly saliva (by sandfly bites or SGH injection) has been proven to have protective effect against *Leishmania* infection on several experimental models. Various authors reported reduction of *L. major* infection in Balb/c and C57BL/6 mice using immunisation by *P. papatasi* saliva (Belkaid *et al*., 1998; Kamhawi *et al.,* 2000), Balb/c mice protection from *L. amazonensis* infection by *L. longipalpis* saliva (Thiakaki *et al.,* 2005). Even humans immunized by *L. longipalpis* bites whose macrophages were protected from infection of *L. chagasi in vitro* (Vinhas et al., 2007). However, it is important that the protective effect was not confirmed in all cases – for example *L. intermedia* saliva did not

protect Balb/c from *L. braziliensis* infection (de Moura *et al.,* 2007), showing the importance of differences in sandfly saliva for vaccine preparation.

The peptide maxadilan from *L. longipalpis* saliva (and PpSP15 from *P. papatasi* saliva in parallel, see below) was the first salivary component shown to exacerbate infection with *L. major* to the same degree as whole saliva and immunizing against maxadilan protected against infection with *Leishmania* – CBA mice were protected against *L. major* infection after immunization with syntetic maxadilan (Morris *et al.*, 2001).

After this success Gomes *et al.* (2008) immunized hamsters with 16 DNA plasmids coding for salivary proteins of *L. longipalpis* and found out that LJM19, an 11-kDa protein with unknown function, protected the hamsters against fatal outcome of VL caused by *L. infantum*. LJM19-immunized hamsters maintained a long-term low parasite load that correlated with the Th1 immune response - an overall high IFN-ɣ/TGF-β ratio and inducible NOS expression were detected in the spleen and liver up to 5 months post infection and a delayed-type hypersensitivity response with high expression of IFN-gamma was also noted in the skin (Gomes *et al.,* 2008). This effect of LJM19 was also confirmed for infection by *L. braziliensis* - immunization with a DNA plasmid coding for LJM19 salivary protein induced protection in hamsters challenged with *L. braziliensis* plus both *L. intermedia* and *L. longipalpis* saliva (Tavares *et al.*, 2011).

Another DNA plasmid coding for yellow-related protein LJM11 maintained short-term (between 2 and 5 months) protection of hamsters against *L. infantum* (Gomes *et al.*, 2008) and at least 12 weeks protective immunity against *L. major* infection in C57BL/6 mice (Xu *et al.*, 2011). However, the effects of *L. longipalpis* proteins on dogs were different: LJM19 induced only weak reaction but LJL143 (unknown function) and LJM17 (yellow-related protein) produced a strong DTH response which showed a consistent systemic adaptive immune response indicative of a Th1 profile. Additionally, SGH-stimulated lymphocytes from immunized dogs efficiently killed *L. infantum chagasi* within autologous macrophages (Collin *et al.*, 2009).

Similarly, PpSP15 protein from *P. papatasi* saliva has provided protection against *L. major* infection in vaccinated mice (Valenzuela *et al.*, 2001a) and after this finding 10 different *P. papatasi* salivary components were tested as vaccine candidates (Oliveira *et al.*, 2008). Mice immunized with cDNA containing two of them – the PpSP15 and yellow-related protein PpSP44 have developed DTH response which is considered to lead to protection

against *Leishmania* infection but only PpSP15 protected mice from *L. major* infection. In contrast, PpSP44-immunized mice aggravated the infection – the explanation is that PpSP15 immunized mice showed distinctly higher Th1 cytokine IFN-ɣ expression and lower Th2 cytokine IL-4 expression than PpSP44 immunized mice (Oliveira *et al.*, 2008).

DNA plasmids coding for ten selected transcripts of *L. intermedia* salivary proteins were constructed and used to immunize BALB/c mice to study their immunogenicity (de Moura *et al.*, 2013). Plasmid Linb-11 coding for a 4.5-kDa protein induced a cellular immune response and conferred protection against *L. braziliensis* infection. This protection correlated with a decreased parasite load and an increased frequency of IFN-ɣ-producing cells (de Moura *et al.*, 2013).

2.3. Salivary antigens detected by sera of bitten hosts

Sandfly salivary components elicit antibody response in the vertebrate host (reviewed in Andrade & Teixeira, 2012). This response is well measurable by the immunologic methods and it plays important role in identifying whether the host is in danger of *Leishmania* transmission (Rohousova *et al.*, 2005). Some studies have even proved positive correlation between anti-saliva antibody response and anti-*Leishmania* (causing visceral disease – *L. chagasi* in South America and *L. infantum* in Europe) DTH without correlation to anti-*Leishmania* antibodies (Gomes *et al.*, 2002; Aquino *et al.*, 2010; Vlkova *et al.*, 2011). For the CL causing *Leishmania* species, positive correlation with the anti-saliva antibodies was found – individuals with active lesions caused by *L. tropica* had higher anti-*P. sergenti* IgG levels compared to healthy individuals, strongly suggesting that production of anti-saliva antibodies could be used as a marker for risk of developing CL (Rohousova *et al.*, 2005). Similar results were found in case of anti-*P. papatasi* antibodies and *L. major* (Marzouki *et al.*, 2011) or anti-*L. intermedia* antibodies and *L. braziliensis* (de Moura *et al.*, 2007). Together with the confirmation that the production of specific antibodies against vector saliva positively correlates with the number of blood fed sandflies (Hostomska *et al.*, 2008, Vlkova *et al.*, 2011), determination of the best antigens is crucial for development of a standard method serving as a disease transmission indicator. Such a monitoring technique would be also useful for evaluating the need for, and effectiveness of, anti-vector campaigns (Gomes *et al*., 2007; Hostomska *et al*., 2008).

Salivary antigens of *L. longipalpis* have been investigated in several studies – they were identified for human sera (Barral *et al.*, 2000; Gomes *et al.*, 2002; Rohousova *et al.*, 2005; Silva *et al.*, 2005; Gomes *et al.*, 2007; Vinhas *et al.*, 2007; Teixeira *et al.*, 2010) and canid sera (Bahia *et al.*, 2007; Gomes *et al.*, 2007; Hostomska *et al.*, 2008; Teixeira *et al.*, 2010). Human sera of individuals who experienced anti–*L. chagasi* serologic status or anti–*L. chagasi* DTH response detected 16 bands by Western blot in the wide range from 200 to 6 kDa (Gomes *et al.*, 2002). Of these, two yellow-related proteins (45 and 35 kDa) and maxadilan (6 kDa) were identified and the yellow-related proteins were the strongest antigens detected by the highest number of sera. This finding was similar to previous reports (Barral *et al.*, 2000 detected bands of 6, 12, 36, and 96 kDa), it was also repeated for the most antigenic proteins - 45, 44, 35 and 16 kD bands (Silva *et al.*, 2005; Vinhas *et al.*, 2007), and identity of the yellow-related proteins and D7-related proteins (29 kDa) was confirmed by mass spectrometry (Bahia *et al.*, 2007). Canine sera from experimentally bitten dogs revealed up to six salivary protein bands with approximate molecular weight of 66, 55, 45 (yellow-related), 37–39 (yellow-related), 34 (apyrase) and 25 kDa (apyrase; Hostomska *et al.*, 2008), while naturally exposed dogs detected 47 kDa yellow-related protein and 29 kDa D7-related protein (Bahia *et al.*, 2007). In a single study, antibodies against *L. longipalpis* saliva were studied also in wild carnivores. Sera of eleven foxes (*Vulpes vulpes)* collected in the endemic area recognized only a few salivary proteins and only one strongly of approximately 50 kDa (Teixeira *et al.*, 2010). Interestingly, *L. longipalpis* antigens were not detected by sera from humans exposed to *P. papatasi* or *P. sergenti* antigens (Rohousova et al., 2005) but some cross-reactions were found between *L. longipalpis* and *L. intermedia* antigens (Teixeira et al., 2010).

Salivary antigens were also determined for eight species of the genus *Phlebotomus* - *P. papatasi* (Volf & Rohousova, 2001; Rohousova *et al.*, 2005; Marzouki *et al.*, 2011)*, P. sergenti* (Rohousova *et al.*, 2005)*, P. tobbi* (Rohousova *et al.*, 2012), *P. pernicious* (Volf & Rohousova, 2001)*, P. halepensis* (Volf & Rohousova, 2001), *P. orientalis* (Vlková *et al.,* 2014), *P. arabicus* (Hostomska *et al.*, 2009) and *P. argentipes* (Martin-Martin *et al.*, 2013a). Among *P. (Phlebotomus) papatasi* and *P. (Paraphlebotomus) sergenti* 14 main bands identified using SDS-PAGE, most were antigenic and reacted with both, mouse and human sera (Rohousova *et al.*, 2005). Differences were, however, found in the intensity of reaction. Mice sera recognized in *P. papatasi* saliva preferentially a 42 kDa protein band. On the other hand, all

positive human sera reacted strongly with the 30 kDa band and the 36 kDa antigen was which was recognized exclusively by human sera. The 30, 36 and 42 kDa protein bands were running to the molecular weights corresponded to D7 protein, apyrase, and Yellow protein, respectively (Valenzuela *et al.* 2001a; Rohousova *et al.*, 2005). The 30 kDa protein was identified by mass spectrometry as D7 protein PpSP30 (Marzouki *et al.*, 2011). In *P. sergenti* SGL the differences were found especially in the intensity of reaction with 12 and 13 kDa polypeptides, the number and reaction intensity of the other antigens was similar to *P. papatasi* but their molecular weight was not exactly same (Rohousova *et al.*, 2005).

About dozen *P. (Larroussius) tobbi* antigens were recognized by sera of experimentally bitten rabbits using immunoblotting and MALDI-TOF: yellow-related proteins (PtSP37 and PtSP38), apyrases (PtSP4 and PtSP10), antigen 5-related proteins (PtSP77 and PtSP79), PpSP32-like proteins (PtSP28 and PtSP29), D7-related proteins (PtSP58 and PtSP60), and PpSP15-like proteins (PtSP9, PtSP23, and PtSP32; Rohousova *et al.*, 2012).

Several studies described salivary antigens of two closely related sandfly subgenera *Larroussius* and *Adlerius*. Sera from rabbits and hamsters experimentally bitten by *P. perniciosus* recognized about 8 major bands and interestingly cross-reacted weakly with 2 or 4 bands from saliva of *P. halepensis* which belongs to subgenus *Adlerius* (Volf & Rohousova, 2001). In the opposite combination, anti-*P. halepensis* sera gave also weak reaction with 1-4 polypeptides from *P. perniciosus* saliva while these sera recognized 4-8 antigens of the homologous antigens from *P. halepensis* - the most prominent ranging from 31 to 42 kDa (Volf & Rohousova, 2001). Mice immunized by exposure to *P. (Adlerius) arabicus* bites were able to recognize 7 strong (56–58.5 kDa, 45 kDa, 43 kDa - a double band, 42 kDa, 34.5–36.5 kDa and 30 kDa) and 4 weak bands (31 kDa, 30.5, 21 and 16 kDa) on immunoblot (Hostomska *et al.*, 2009). On the other hand, no reaction was found among antigens from a representative of *Adlerius* species and *Phlebotomus* and *Paraphlebotomus* species - *P. (Paraphlebotomus) sergenti* recognized only homologous antigens but no heterologous antigens from *P. (Adlerius) arabicus* or *P. (Phlebotomus) papatasi* (Drahota *et al*., 2009).

Very little is known about intraspecific variability of salivary proteins. In two colonies of *P. (Larroussius) orientalis* originating from two different parts of Ethiopia (AZ – Addis Zemen and MW – Melka Werer) very similar antigens detected with mice sera: the most intensive reactions detected the yellow-related proteins (AZ: PorASP2, PorASP4; MW: PorMSP23, PorMSP24), apyrases (AZ: PorASP11, Por-ASP14, PorASP15; MW: PorMSP3,

PorMSP4), and antigen 5-related proteins (AZ: PorASP74, PorASP76; MW: PorMSP6, PorMSP8). All these proteins were recognized by all mice sera tested, while D7-related proteins (AZ: PorASP48, PorASP122; MW: PorMSP28, PorMSP38, PorMSP67) and PpSP15-like proteins (AZ: PorASP28, PorASP37, PorASP61; MW: PorMSP12, PorMSP74, PorMSP96) were recognized only by some sera (Vlkova *et al.*, 2014).

Another important vector of visceral leishmaniases is *P. (Euphlebotomus) argentipes*. Sera of laboratory hamsters bitten by this species recognized at least 20 *P. argentipes* salivary protein spots after 2DE-Western blot separation followed by MALDI-TOF identification - three of them were identified as PpSP15-like proteins (SP01, 02, 07), one as apyrase (SP03), one as antigen 5-related protein (SP05), one as D7- related protein (SP10) and five proteins with unknown function (SP06, SP09, SP17, SP20 and SP56; Martin-Martin *et al.*, 2013).

2.4. Recombinants proteins from sandfly saliva as markers of host exposure

Single sandfly salivary proteins have been proven to elicit host antibody response in two model situations – as DNA plasmids developed for vaccination and as recombinant proteins prepared as markers of host exposure to sandfly bites. As mentioned above, the DNA vaccines were developed based on protein sequences from *P. papatasi* (Valenzuela *et al*., 2001a; Oliveira *et al*., 2008), *L. longipalpis* (Morris *et al.,* 2001; Gomes *et al*., 2008; Collin *et al.*, 2009; Tavares et al., 2011; Xu *et al*., 2011) and *L. intermedia* (de Moura *et al*., 2013). Mice immunized with DNA plasmid containing sequence of *P. papatasi* PpSP15 protein generated both cellular and humoral immunity response – on western blots prepared from *P. papatasi* SGH, 15 kDa band reacted with the immunized mice sera (Valenzuela *et al.*, 2001). Among the sixteen plasmids tested - each containing sequence of one protein from *L. longipalpis* saliva, six induced antibody response - maxadilan (LJL08), D7-related protein (LJL13), yellow-related proteins (LJM111, 11, 17) and 5´nucteotidase (LJL11); two of the yellow-related proteins – LJM11, 17 induced the strongest antibody response and they also lead to DTH in hamsters (Gomes *et al.*, 2008). For *L. intermedia* which saliva is associated with the risk of acquiring *L. braziliensis* infection in humans (de Moura *et al.*, 2007), ten protein sequences were used for mice immunization and all of them induced humoral immune response (de Moura *et al.*, 2013). These proteins belong to five protein families – SP13 protein family, SP15-like proteins, C-type lectin family, 9.6-kDa proteins and 10-kDa

proteins, and as mentioned above only one plasmid (Linb-11) - coding for a 4.5-kDa protein belonging to the SP13 protein family, induced a cellular immune response and conferred protection against *L. braziliensis* infection (de Moura *et al.*, 2013).

Within the two most frequently studied species - *L. longipalpis* and *P. papatasi*, recombinant proteins have been produced in bacterial expression system and their antigenicity was tested (Teixeira *et al.*, 2010; Souza *et al.*, 2010; Marzouki *et al.*, 2012). Nine most antigenic *L. longipalpis* salivary proteins (Gomes *et al.*, 2002; see above) were expressed and tested – three of them belong to the yellow-related protein family (LJM11 – 45 kDa, 17 – 43.2 kDa, 111 – 43 kDa), one is an apyrase (LJL23 – 35.1 kDa), one belong to D7-related proteins (LJL13 – 26.5 kDa), one is from PpSP15 or SL1 protein family (LJM04 – 13.8 kDa), one is endonuclease (LJL138 – 43.7 kDa), one is 5'nucleotidase (LJL11 – 60.5 kDa) and the last has an unknown function (LJL143 – 32.4 kDa) (Teixeira *et al.*, 2010). Compared to vaccination with six *L. longipalpis* plasmids leading to humoral immune response in hamsters (Gomes *et al.*, 2008), in humans (20 individuals), dogs (8 individuals) and foxes (11 individuals) from *L. longipalpis* endemic regions, the yellow-related protein LJM17 was confirmed to be the most antigenic as it was the only one recognized by all three host species. This finding confirms the previous results that antibodies against *L. longipalpis* saliva in the fox *Cerdocyon thous* detect primarily a 44 kDa protein (Gomes *et al.*, 2007). The other yellow-related protein strongly recognized by hamsters – LJM11, was recognized well also by both human and dog sera (Teixeira *et al.*, 2010). These two proteins were tested in next study by ELISA with another 40 human sera from individuals who were seropositive and 40 sera seronegative to *L. longipalpis* SGS (Souza *et al.*, 2010). This screening has shown that each recombinant protein was able to detect anti-saliva seroconversion, whereas the two proteins combined increased the detection significantly, and the specificity of the anti-*L. longipalpis* response evaluated by testing 40 sera positive to *L. intermedia* SGS, and very limited (2/40) cross-reactivity was observed. Predicted threshold levels were confirmed for LJM17+LJM11 using another 1,077 serum samples (Souza *et al.*, 2010). Of the other seven *L. longipalpis* recombinant proteins tested by Teixeira *et al.* (2010), the third yellow-related protein, LJM111 (43 kDa) was only recognized by human sera. LJL23 (35 kDa), LJL13 (26.5 kDa), and LJM04 (13.8 kDa) proteins were recognized only by dog sera; LJL143 was recognized by dog sera and weakly recognized by human sera. LJL11 and LJL138 were not recognized by any of the sera tested (Teixeira *et al.*, 2010).

In *P. papatasi*, the 30 kDa salivary antigen previously identified as D7-related protein PpSP30 (Marzouki *et al.*, 2011) was expressed together with PpSP32 protein in bacteria and tested with human and mice sera (Marzouki *et al.*, 2012). Surprisingly, recombinant PpSP30 was poorly recognized by human sera from areas endemic for *L. major* and it did not induce any detectable levels of IgG antibodies in mice, while PpSP32 was strongly recognized by the both human and mice sera (Marzouki *et al.*, 2012).

Another interesting finding has been achieved with *L. ayacuchensis* – thanks to the transcriptome data, T cell epitopes were predicted for future best antigens synthesis (Kato *et al.*, 2013). HLA class II-binding peptides were searched on the 51 different HLA-DR alleles, and the promiscuous epitopes were selected from the *L. ayacuchensis* salivary protein sequences of proteins tested that were predicted to bind at least 20 alleles. Four potential epitopes from SP15-like and twelve from yellow-related protein sequences were found (Kato *et al.*, 2013).

In conclusion, recombinant salivary proteins or plasmids coding for salivary antigens were studied in a limited number of sandfly species. Therefore, the main aims of these PhD theses were to identify the major salivary antigens of two important European sandflies, *P. perniciosus* and *P. papatasi*, prepare recombinant forms of these antigens and then test them with sera of hosts bitten by these sandfly species.

Objectives

Previous laboratory experiments revealed that antibodies against saliva of sand flies reflect the number of sand fly bites and therefore anti-saliva IgG can serve as a marker of exposure to sand flies. Another interesting aspect of the anti-saliva antibody level is its correlation with susceptibility or immunity to *Leishmania* parasites – that means that in VL cases high antibody levels confer immunity to *Leishmania* infection but in CL cases, in the opposite, high antibody levels serve as a marker for risk of developing leishmaniases.

The first aim of the study was to test antibody response to *P. perniciosus* saliva in naturally exposed host and identify the major salivary antigens of *P. perniciosus*. We tested, for the first time in the Old World, the anti-saliva IgG in sera of dogs living in area endemic for *L. infantum*. We received interesting results but also found that the use of whole salivary gland lysate is very laborious and time consuming and this is why we decided to replace whole saliva by selected salivary antigens made in recombinant form.

The second and major aim of the theses was, therefore, to use recombinant salivary proteins (rSPs) of sand flies as markers of exposure. We were interested to study if this new concept is doable and usefull. We choose two European vectors with already constructed cDNA sialomes – *P. perniciosus* and *P. papatasi,* and identify and then prepare in recombinant form their major salivary antigens. Then we tested antigenicity of these rSPs by various immunologic methods.

We decided to develop this method on laboratory model *P. papatasi* – Balb/c mice where we optimized the expression system and detection methods. Then we applied these methods on sera of hosts bitten by *P. perniciosus*. After testing these antigens on the laboratory mice model, the final aim was to conduct a study with the natural reservoirs of *L. infantum* - dogs, hares, and wild rabbits.

Publications

1. Vlkova M, Rohousova I, **Drahota J**, Stanneck D, Kruedewagen EM, Mencke N, Otranto D, Volf P (2011). Canine antibody response to Phlebotomus perniciosus bites negatively correlates with the risk of Leishmania infantum transmission. PloS Neglected Tropical Diseases 5: e1344.

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Canine Antibody Response to *Phlebotomus perniciosus* Bites Negatively Correlates with the Risk of *Leishmania infantum* Transmission

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Abstract

Background: Phlebotomine sand flies are blood-sucking insects that can transmit *Leishmania* parasites. Hosts bitten by sand flies develop an immune response against sand fly salivary antigens. Specific anti-saliva IgG indicate the exposure to the vector and may also help to estimate the risk of *Leishmania* spp. transmission. In this study, we examined the canine antibody response against the saliva of *Phlebotomus perniciosus*, the main vector of *Leishmania infantum* in the Mediterranean Basin, and characterized salivary antigens of this sand fly species.

Methodology/Principal Findings: Sera of dogs bitten by *P. perniciosus* under experimental conditions and dogs naturally exposed to sand flies in a *L. infantum* focus were tested by ELISA for the presence of anti-*P. perniciosus* antibodies. Antibody levels positively correlated with the number of blood-fed *P. perniciosus* females. In naturally exposed dogs the increase of specific IgG, IgG1 and IgG2 was observed during sand fly season. Importantly, *Leishmania*-positive dogs revealed significantly lower anti-*P. perniciosus* IgG2 compared to *Leishmania*-negative ones. Major *P. perniciosus* antigens were identified by western blot and mass spectrometry as yellow proteins, apyrases and antigen 5-related proteins.

Conclusions: Results suggest that monitoring canine antibody response to sand fly saliva in endemic foci could estimate the risk of *L. infantum* transmission. It may also help to control canine leishmaniasis by evaluating the effectiveness of anti-vector campaigns. Data from the field study where dogs from the Italian focus of *L. infantum* were naturally exposed to *P. perniciosus* bites indicates that the levels of anti-*P. perniciosus* saliva IgG2 negatively correlate with the risk of *Leishmania* transmission. Thus, specific IgG2 response is suggested as a risk marker of *L. infantum* transmission for dogs.

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Introduction

Leishmania infantum (syn. *Leishmania chagasi*) is a protozoan parasite that causes zoonotic leishmaniasis, including the life-threatening visceral form, occurring also in the Mediterranean Basin. Parasites are transmitted by the bite of infected phlebotomine sand flies to dogs, the major host and the main domestic reservoir for human visceral leishmaniasis, or to humans. The clinical forms of canine leishmaniasis range from asymptomatic to lethal (reviewed in [1,2]). Nonetheless, all seropositive infected dogs, including those without any clinical signs, can serve as a source of infection for sand flies in endemic areas [3,4]. The major vector of canine leishmaniases in Mediterranean countries, including Italy, is *Phlebotomus perniciosus* [5,6]. Control programs for human visceral leishmaniasis caused by *L. infantum* are primarily aimed at preventing sand flies from feeding on dogs to reduce *Leishmania* transmission among dogs and humans (reviewed in [1,2]).

Measuring the exposure of dogs to sand fly bites is important for estimating the risk of *L. infantum* transmission. Recently, it was demonstrated that experimental exposure of dogs to *Lutzomyia longipalpis* bites elicits the production of specific anti-saliva IgG which positively correlates with the number of blood-fed sand flies [7]. Therefore, monitoring canine IgG levels specific for sand fly saliva could indicate the intensity of exposure to sand fly bites. Such a monitoring technique would be useful for evaluating the need for, and effectiveness of, anti-vector campaigns [7,8].

Exposure to sand fly bites as well as immunization with sand fly saliva or its compounds elicits in naive hosts protection against *Leishmania* infection under laboratory conditions (reviewed in [9]). It is widely accepted that the protective effect is mediated by $CD4^+$ Th1 cellular response and characterized by increased production of IFN- γ, which activates macrophages to kill *Leishmania* parasites (reviewed in [10]). Recently, it was shown

Author Summary

Leishmania infantum is the causative agent of zoonotic visceral leishmaniasis in the Mediterranean Basin and *Phlebotomus perniciosus* serve as the major vector. In the endemic foci, *Leishmania* parasites are transmitted mostly to dogs, the main reservoir host, and to humans. We studied the canine humoral immune response to *Phlebotomus perniciosus* saliva and its potential use as a marker of sand fly exposure and consequently as a risk marker for *Leishmania* transmission. We also characterized major salivary antigens of *P. perniciosus*. We demonstrated that under laboratory conditions, the levels of anti-*P. perniciosus* saliva antibodies positively correlated with the number of blood-fed sand flies and therefore, may be used to evaluate the need for, and the effectiveness of, anti-vector campaigns. In parallel, we studied sera of dogs naturally exposed to *P. perniciosus* in highly active focus of canine leishmaniasis in Southern Italy. Specific antibodies against *P. perniciosus* saliva were significantly increased according to the ongoing sand fly season. Moreover, the levels of anti-*P. perniciosus* antibodies in naturally bitten dogs negatively correlated with anti-*Leishmania* seropositivity. Thus, for dogs living in endemic areas, specific antibody response against saliva of the vector is an important marker for estimating the risk of *Leishmania* transmission.

that protective effect elicited by inoculation of *Lutzomyia longipalpis* recombinant proteins in dogs was associated with production of IFN-γ by $CD3^+$ $CD4^+$ T cells and by dominance of IgG2 antibodies [11].

In this study we described the anti-saliva IgG response in dogs experimentally exposed to *P. perniciosus* under laboratory conditions and those naturally exposed in an endemic focus of *L. infantum*. We also tested the association between the anti-saliva IgG subclasses and the levels of IFN-γ in *Leishmania infantum*-seropositive and -seronegative dogs. Additionally, we characterized the major *P. perniciosus* salivary antigens recognized by sera of experimentally and naturally bitten dogs.

Methods

Ethical statement

Experiments with dogs exposed to sand fly bites under laboratory conditions. Husbandry of animals in the Animal Center (Germany) complies with the European Commission guidelines for the accommodation of animals used for experimental and other scientific purposes - Commission Recommendation of 18 June 2007 (2007/526/EC). The compliance to aspects of animal welfare law is regularly monitored by the BAH animal welfare commissioner and the state veterinarian. The study design and the experimental procedures were approved by the responsible authorities (LANUV - Regional Authority for Nature, Environment and Consumer protection in North Rhine-Westphalia, Germany).

Experiments with dogs naturally exposed to sand fly bites. All procedures were approved by the Animal Ethics Committee from the Faculty of Veterinary Medicine, University of Bari, Italy and authorized by the Italian Ministry of Health (Authorization number 72/2009C n°69062; 28/11/08). Adverse events were individually registered in accordance to the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) and Good Clinical Practice (GCP) Guideline (GL9).

Sand flies and salivary gland dissection

A colony of *Phlebotomus perniciosus* was reared under standard conditions as described in [12]. Salivary glands were dissected from 4–6 day old female sand flies, placed into 20 mM Tris buffer with 150 mM NaCl and stored at $-20^{\circ}C$.

Experimental exposure

Twelve laboratory dogs, beagles, were housed and handled in the Bayer Animal Health GmbH animal facility (Leverkusen, Germany). Dogs were sedated and individually exposed to approximately 200 *P. perniciosus* females as described in [7,13]. Twenty hours after exposure, sand flies were collected and microscopically examined to assess the ratio of blood-fed females. In two independent experiments, two groups of three dogs each were used. Dogs in groups 2 and 4 wore insecticide-impregnated collars that were administrated 8 days before the first sand fly exposure, for a reduction of sand fly bites. In comparison, dogs in groups 1 and 3 remained without any repellent or insecticide application during the whole study. Therefore, dogs in groups 1 and 3 are hereafter defined as high-exposed (HE) and the dogs in groups 2 and 4 as low-exposed (LE). Dogs were exposed to sand fly bites once a week for five consecutive weeks. For the detailed numbers of blood-fed females see Table 1. Blood samples were collected throughout the study according to the following schedule: before the first exposure (week 0, pre-immune serum), during the sand fly sensitization (weeks 1–5), and weekly after the last exposure for 5 weeks (weeks 6–10).

Field study

Twenty nine mixed-breed young dogs (from 90 to 145 days old) and eleven laboratory reared beagles (120 days old) were enrolled in the trial. All animals were housed in a private open-air shelter in Putignano (Bari province, Apulia, Italy), where *P. perniciosus* is the most abundant phlebotomine sand fly species [14]. All dogs were vaccinated against common dog pathogens and dewormed as described in [15]. The canine antibody response against *P. perniciosus* saliva was studied at the beginning (March 2008) and at the end (November 2008) of the sand fly season. In parallel, at four intervals (March, July, November 2008 and March 2009) dogs were tested for *L. infantum* infection status by serological, cytological and molecular methods. All dogs were *L. infantum* negative at the beginning of the trial (March 2008), which was proved by all three diagnostic methods used. *Leishmania*-positive dogs were defined by positive anti-*L. infantum* serology and, in a subset of seropositive dogs (4 out of 18), the infection was confirmed by PCR or cytology. For details on the diagnostic

Table 1. Numbers of blood-fed *Phlebotomus perniciosus* females per dog.

Week	Group 1	Group 2	Group 3	Group 4
1	221±5	49±15	173±8	27±4
2	191±47	125±69	155±18	11±6
3	188±7	61±20	125±6	36±15
4	156±4	39±11	169±12	20±3
5	195±9	83±36	158±11	8±1
average	190±10	71±16	156±6	20±4

(average ± standard error; groups 1, 3 – high-exposed dogs; groups 2, 4 – low-exposed dogs).
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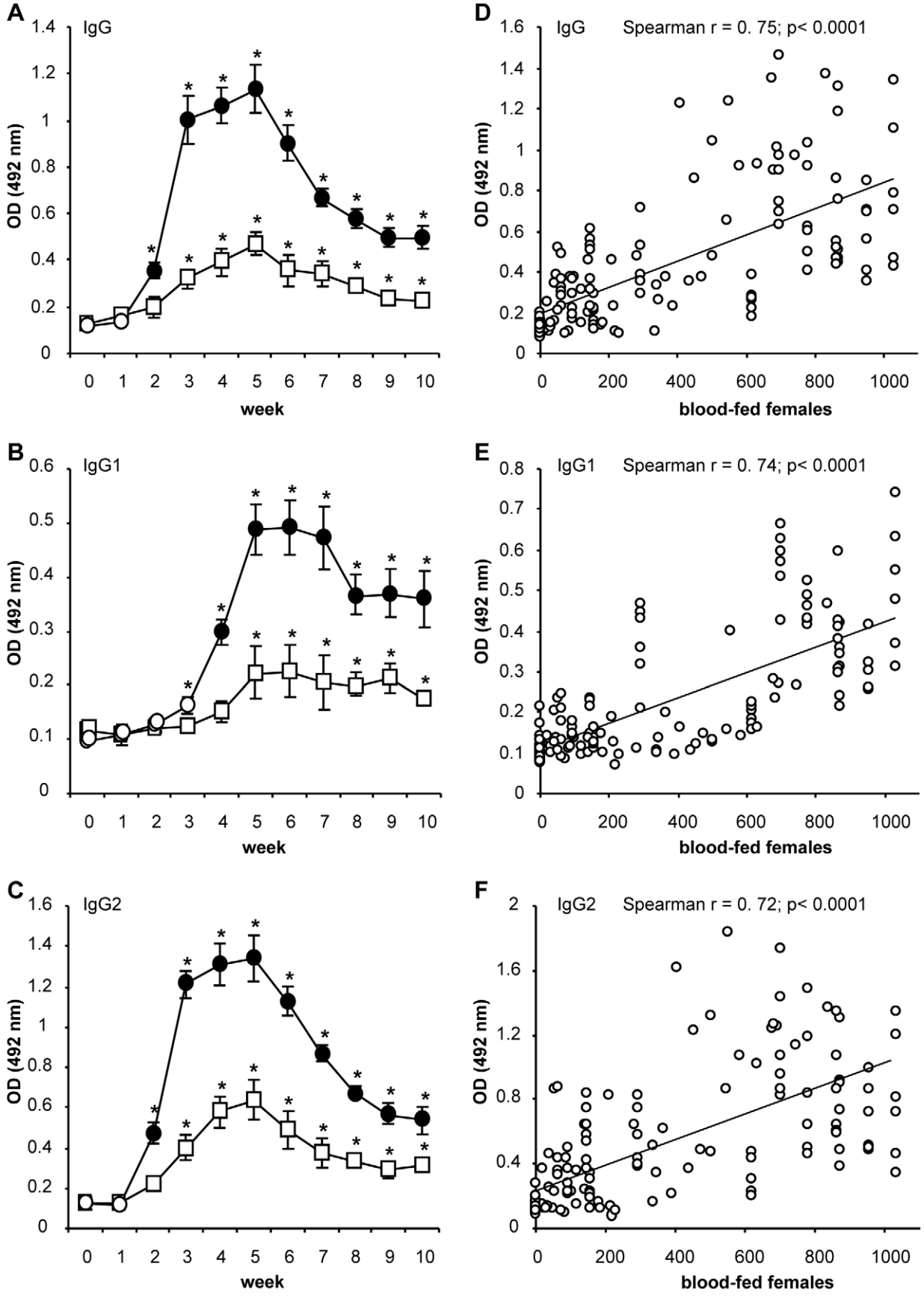
A
IgG
OD (492 nm)
week
B
IgG1
OD (492 nm)
week
C
IgG2
OD (492 nm)
week
D
IgG
Spearman r = 0. 75; p< 0.0001
OD (492 nm)
blood-fed females
E
IgG1
Spearman r = 0. 74; p< 0.0001
OD (492 nm)
blood-fed females
F
IgG2
Spearman r = 0. 72; p< 0.0001
OD (492 nm)
blood-fed females

Figure 1. Anti-sand fly saliva antibody response in dogs experimentally bitten by *Phlebotomus perniciosus*. (A–C) Beagle dogs (3 per group) were divided into low-exposed (square) and high-exposed groups (circles) and were exposed to sand fly bites in weeks 1–5. For detailed numbers of blood-fed females see Table 1. Levels of specific IgG (A); IgG1 (B); and IgG2 (C) were measured by ELISA (at 492 nm) in all canine pre-immune and immune sera. Full circles represent significant difference between high- and low-exposed dogs ($p<0.05$); asterisks indicate significant difference ($p<0.05$) compared to pre-immune sera. Data are presented as the means ± standard errors of the means from two independent studies. (D–F) Correlation between number of blood-fed sand fly females and the levels of canine anti-*P. perniciosus* IgG (D); IgG1 (E); and IgG2 (F) was performed using Spearman Rank Correlation Matrix. OD = optical density.
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methods, see [15,16]. Considering the long incubation period of canine leishmaniasis and the occurrence of sand flies exclusively during the summer season (from June to October) [14], dogs with anti-*Leishmania* seroconversion in March (2009) are presumed to have become infected during the previous season (2008). Dogs that were seronegative for *L. infantum* at all four screening intervals were included in the *Leishmania*-negative group.

Detection of anti – *P. perniciosus* saliva antibodies

Anti-*P. perniciosus* IgG, IgG1 and IgG2 were measured by enzyme-linked immunosorbent assay (ELISA) as described in [7] with some modification. Briefly, microtiter plates were incubated with 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine sera were diluted 1:200 or 1:500 in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, IgG1, or IgG2 from Bethyl laboratories) were diluted and incubated as previously described [7]. Absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). The cut-off value (IgG = 0.145; IgG1 = 0.126; IgG2 = 0.165) was determined as less than two times the standard error of the mean of the absorbance of pre-immune serum.

Western blot analysis

Phlebotomus perniciosus salivary gland homogenate from 5-day-old sand fly females were separated by SDS-PAGE on a 10% gel under non-reducing conditions using the Mini-Protean III apparatus (BioRad). Separated proteins were blotted onto a nitrocellulose (NC) membrane by Semi-Phor equipment (Hoefer Scientific Instruments) and blocked with 5% (w/v) low fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-Tw). Strips of NC membrane were incubated with canine sera diluted 1:50 (experimentally bitten dogs) or 1:25 (naturally bitten dogs) in TBS-Tw for 1 hour. The strips were then washed three times with TBS-Tw and incubated with peroxidase-conjugated sheep anti-dog IgG (Bethyl Laboratories) diluted 1:3000 in TBS-Tw. The chromogenic reaction was developed using a solution containing diaminobenzidine and H_2O_2.

Mass spectrometry

For mass spectrometric analysis, salivary glands from 5-day-old *P. perniciosus* females were homogenized by 3 freeze-thaw cycles. Samples were dissolved in non-reducing sample buffer and electrophoretically separated in 10% polyacrylamide SDS gel. Proteins within the gels were visualized by staining with Coomassie Blue G-250 (Bio-Rad). The individual bands were cut and incubated with 10 mM dithiothreitol (DTT) and then treated with 55 mM iodoacetamid. Washed and dried bands were digested with trypsin (5 ng Promega). The alpha-cyano-4-hydroxycinnamic acid was used as a matrix. Samples were measured using a 4800 Plus MALDI TOF/TOF analyzer (AB SCIEX). Peak list from the MS spectra was generated by 4000 Series Explorer V 3.5.3 (AB SCIEX) without smoothing. Peaks with local signal to noise ratio greater than 5 were picked and searched by local Mascot v. 2.1 (Matrix Science) against a database of putative salivary protein sequences derived from a cDNA library [17]. Database search criteria were as follows – enzyme: trypsin, taxonomy: *Phlebotomus*, fixed modification: carbamidomethylation, variable modification: methionine oxidation, peptide mass tolerance: 80 ppm, one missed cleavage allowed. Only hits that scored as significant ($p<0.05$) are included.

Statistical analysis

The data from experimentally bitten dogs obtained by ELISA were subjected to GLM ANOVA and Scheffe's Multiple Comparison procedure to analyse differences in kinetics of antibody response between HE and LE dogs at all sampling points. The non-parametric Wilcoxon rank sum test for differences in medians was used for comparison of anti-*P. perniciosus* IgG, IgG1, IgG2 and IgG1/IgG2 ratios between *Leishmania*-seropositive and -seronegative dogs. The non-parametric Wilcoxon signed-rank test for differences in medians was used for comparison of antibody increases between March and November blood samples in naturally bitten dogs. For correlation tests we used the non-parametric Spearman rank correlation matrix. For all tests statistical significance was regarded as a p-value less than or equal to 0.05. All statistical analyses were performed using NCSS 6.0.21 software.

Relative risk (the probability of the developing the disease occurring in the group exposed to the risk factor versus a non-exposed group), attributive risk (absolute effect of exposure to the risk factor) and ODDS ratio (odds of an event occurring in the exposed group to the odds of it occurring in non-exposed group) were calculated for dogs from the field study to find out the relationship between the levels of anti-*P. perniciosus* saliva antibodies and leishmaniasis incidence as described in [18]. Low level of specific antibodies (lower than the cut-off value) was determined as the risk factor and the confidence interval for relative risk was calculated as described in [19].

List of the protein accession numbers

Phlebotomus perniciosus: DQ153102; DQ154099; DQ150622; DQ150621; DQ192490; DQ192491; DQ153100; DQ153101; DQ153104; DQ150624; DQ150623; DQ150620; DQ153105.

Lutzomyia longipalpis: AF132518.

Results

Antibody response in experimentally bitten dogs

To investigate the kinetics of antibody response against anti-*P. perniciosus* saliva, two groups of experimentally bitten dogs, low-exposed (LE) and high-exposed (HE), were followed for 10 weeks. Five weekly experimental exposures to *P. perniciosus* bites led to increased levels of anti-saliva specific IgG, IgG1 and IgG2 in both LE and HE groups. No anti-saliva antibodies were detected in any pre-immune dog sera tested.

In HE dogs, anti-*P. perniciosus* antibody levels increased significantly ($p<0.05$) in comparison to the pre-immune sera after the second (IgG; IgG2) and third exposure (IgG1) (Figure 1A–C). Anti-saliva IgG and IgG2 developed with similar kinetics; rapidly increased after the third exposure, and gradual increase until week five (the last exposure), followed by a steady decrease to the end of

the study. Anti-saliva IgG1 increased rapidly between weeks three and five and persisted at elevated levels until the end of the study.

In LE dogs, anti-*P. perniciosus* antibody levels increased significantly ($p<0.05$) in comparison to the pre-immune sera after the fourth (IgG; IgG2) and sixth exposure (IgG1) (Figure 1A–C). Similar to HE dogs, kinetics of anti-*P. perniciosus* IgG and IgG2 in LE dogs was detected at peak levels on week five followed by a rapid decrease. Conversely, IgG1 was measured at peak levels on week six and persisted at elevated quantities to the end of the study (Figure 1A–C).

All HE dogs produced significantly higher levels of anti-*P. perniciosus* IgG ($p = 0.0001$), IgG1 ($p = 0.0032$) and IgG2 ($p = 0.0003$) compared to LE dogs throughout the study (Figure 1A–C). A positive correlation was detected between number of blood-fed female sand flies and the levels of canine anti-*P. perniciosus* IgG ($r = 0.75$, $p<0.0001$), IgG1 ($r = 0.74$, $p<0.0001$) and IgG2 ($r = 0.72$, $p<0.0001$) (Figure 1D–F). Overall, sera of experimentally bitten dogs produced higher concentrations of specific IgG2 compared to specific IgG1 (data not shown).

Antibody response in naturally bitten dogs

To determine the anti-*P. perniciosus* saliva antibody levels and the seasonal changes in specific antibody response, canine sera were screened at the beginning and at the end of the sand fly season, March and November, respectively. Incidence of leishmaniasis in dogs naturally exposed to sand flies was high, 18 out of 40 (45%) were found anti-*L. infantum* seropositive (0/40 in March 2008; 0/40 in July 2008; 5/40 in November 2008; 13/40 in March 2009). In March, higher levels of anti-*P. perniciosus* IgG and IgG2 (compared to cut-off value) were detected in about 55% and 10% of dog sera, respectively, while IgG1 levels were comparable to pre-immune sera (Table 2). In November, elevated levels of specific IgG were found in 87.5%, IgG2 in 72.5% and IgG1 in 45% of the 40 enrolled dogs (Table 2). In both groups of dogs, *Leishmania*-positive and *Leishmania*-negative, specific IgG, IgG1 and IgG2 levels significantly increased during the sand fly season (Figure 2A–C).

Leishmania-positive and *Leishmania*-negative dogs did not statistically differ in IgG and IgG1 production (Figure 2A, B); however, a significant difference was found in IgG2 levels (Figure 2C). Indeed, *Leishmania*-positive dogs revealed significantly lower anti-*P. perniciosus* IgG2 at the beginning ($p = 0.047$) and at the end ($p = 0.05$) of sand fly season (Figure 2C). Negative correlation was found between the levels of anti-*P. perniciosus* saliva IgG2 and the risk of *Leishmania* transmission, supported well by epidemiological parameters: relative risk = 2.6 (95% confidence interval: 0.66; 10.63); attributive risk = 1.6; and ODDS ratio = 10. Sera of all naturally bitten dogs showed significantly higher levels of specific IgG2 compared to specific IgG1 (data not shown). Moreover, the IgG1/IgG2 ratio differed between *Leishmania*-positive and -negative dogs; *Leishmania*-positive dogs revealed higher IgG1/IgG2 ratio, although the difference was statistically significant only at the beginning of sand fly season ($p = 0.039$) (Table 2). Furthermore, higher levels of IFN-γ were detected in sera of *Leishmania*-negative dogs throughout the study but with no statistically significant difference (Figure S1).

Identification and characterization of *P. perniciosus* salivary antigens

Phlebotomus perniciosus salivary antigens were studied using sera of naturally and experimentally bitten dogs. Pre-immune sera of experimentally bitten dogs did not recognize any of the salivary proteins by Western blot analysis (Figure 3).

Sera of experimentally exposed dogs produced 11 bands on a salivary gland Western blot with approximate molecular weights of 75, 50, 42, 40, 38, 34, 33, 29, 27, 23 and 14 kDa (Figure 3). The molecular weights of salivary antigens recognized by canine sera were similar in all dogs tested with the exception of the 23 and 27 kDa protein bands (recognized only by some sera). The salivary gland antigens most intensely recognized by the sera of all experimentally bitten dogs had molecular weights of 42, 38, 33 and 29 kDa.

Sera of naturally bitten dogs with both negative and positive anti-*L. infantum* serology reacted with up to 9 protein bands of 50, 42, 38, 34, 33, 29, 27, 23 and 14 kDa. All naturally exposed dogs tested in both groups recognized similar salivary antigens and the most intensive reactions were detected with the 42 and 33 kDa salivary antigens.

Mass spectrometry revealed that the main antigens recognized by sera of bitten dogs were salivary endonuclease (50 kDa - DQ154099), yellow proteins (42 kDa - DQ150622; 40 kDa - DQ150621), apyrases (38 kDa - DQ192490; 38 kDa - DQ192491; 33 kDa - DQ192491), antigen-5 protein (29 kDa - DQ153101), D7 proteins (27 kDa - DQ153104; 23 kDa - DQ150624; 23 kDa - DQ150623, and proteins of the SP-15 like protein family (14 kDa - DQ150620; 14 kDa - DQ153105) (Table 3).

Discussion

Canine antibody response against *P. perniciosus* saliva was studied in dogs bitten by sand flies under well-defined laboratory conditions as well as in dogs from an endemic focus of visceral leishmaniasis in Italy.

In experimentally bitten dogs we observed a significant increase in production of specific IgG, IgG1 and IgG2 in the course of 10 weeks and a positive correlation was found between the levels of specific antibodies and the number of blood-fed females *P. perniciosus*. Anti-saliva specific IgG and IgG2 developed with similar

Table 2. Numbers of dogs positive for anti-*Phlebotomus perniciosus* antibodies in *Leishmania infantum*-seropositive and -seronegative dogs.

	Leishmania negative dogs (n = 22)			Leishmania positive dogs (n = 18)		
	March	November	Increase(%)	March	November	Increase (%)
IgG	14	19	144***	8	15	104**
IgG1	0	11	235***	0	7	220**
IgG2	2	20	249***	2	9	205***
IgG1/IgG2[a]	0.47*	0.54	15	0.57*	0.73	28

([a] – significant difference in IgG1/IgG2 ratio between *Leishmania*-seropositive and -seronegative groups; *** $p<0.001$; ** $p<0.01$; * $p<0.05$).

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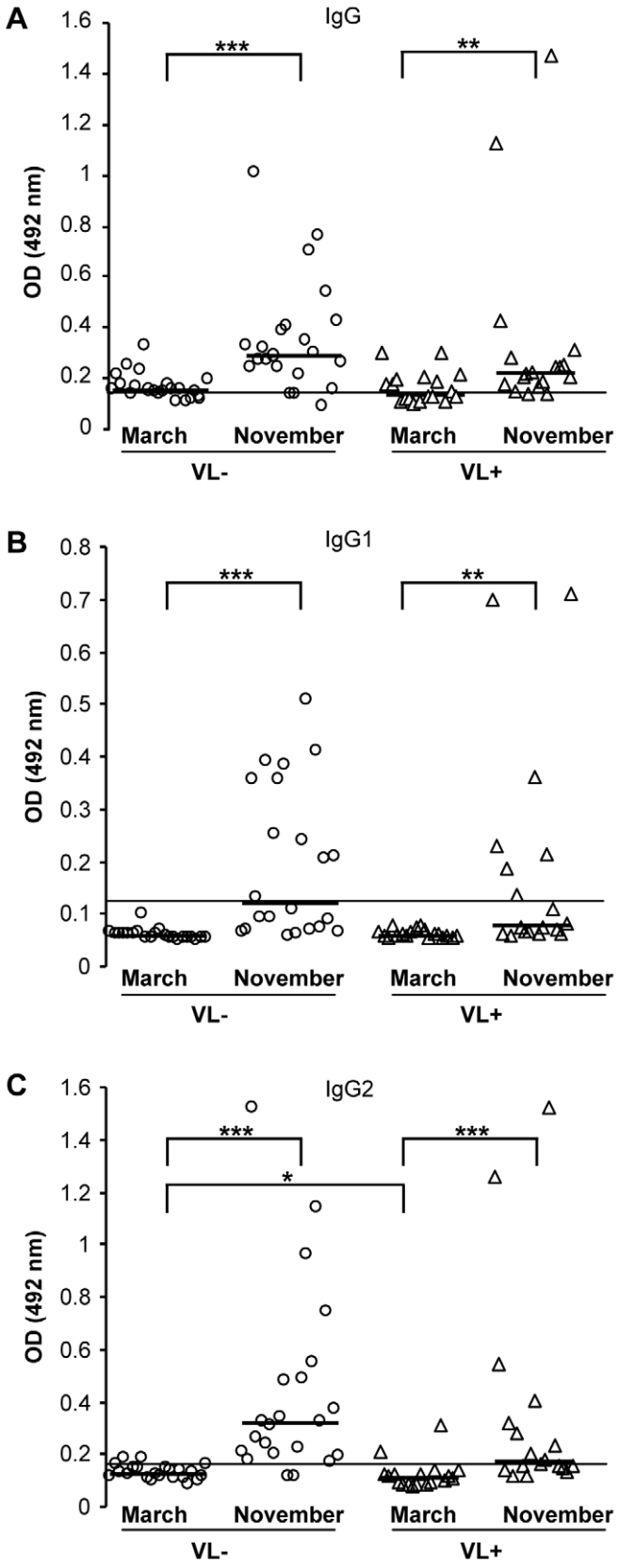

Figure 2. Anti-sand fly saliva antibody response in dogs naturally bitten by *Phlebotomus perniciosus*. Anti-*P. perniciosus* IgG (A); IgG1 (B) and IgG2 (C) response was measured in sera of naturally bitten dogs from endemic area of visceral leishmaniasis. All dogs were *Leishmania infantum* seronegative at the beginning of the trial. ELISA was performed against *P. perniciosus* salivary gland homogenate using canine sera from *Leishmania infantum*-seropositive dogs (open triangle, n = 18) and *Leishmania*-seronegative dogs (open circles, n = 22). Serum samples were taken at the

beginning (March) and at the end of the sand fly season (November). The symbols indicate results of each serum tested, bars represent median values of the groups. Lines represent cut-off values (two times the standard error of the mean of the absorbance of experimentally bitten dog pre-immune sera). Asterisks indicate statistical significance between *Leishmania*-seropositive and -seronegative dogs and significant increase of antibodies during the sand fly season within the group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). OD = optical density.
doi:10.1371/journal.pntd.0001344.g002

kinetics and correspond well with previous results [7] in dogs experimentally bitten by *Lutzomyia longipalpis*. While in sera of healthy dogs, IgG1 and IgG2 usually occur in comparable concentrations [20], IgG2 prevailed in sera of bitten dogs in our study as well as in dogs experimentally bitten by *L. longipalpis* [7,11].

In our field trial, we detected the increase in number of anti-*P. perniciosus* saliva seropositive dogs as well as in the amount of specific antibodies in dog sera as the sand fly season progressed. Statistically significant increases in production of specific IgG, IgG1 and IgG2 were observed in both *Leishmania*-positive and *Leishmania*-negative dogs at the end of sand fly season. Interestingly, *Leishmania*-positive dogs revealed significantly lower anti-*P. perniciosus* saliva IgG2 compared to *Leishmania*-negative dogs and the IgG1/IgG2 ratio was significantly higher in *Leishmania*-positive dogs. These data may suggest either that dogs with low IgG2 levels were at the higher risk of becoming *Leishmania*-infected or that *Leishmania* infection decreases the production of IgG2 in bitten dogs. Considering the IFN-γ levels in canine sera, that were shown to positively correlate with the protective Th1 immune response [11], it seems that the first hypothesis is more feasible. Although, the difference in IFN- γ production between *Leishmania*-negative and *Leishmania*–positive dogs was not statistically significant.

Published data from field studies suggests that humoral immune responses against sand fly saliva vary between hosts with cutaneous and visceral forms of leishmaniases (reviewed in [9,21]). In foci of cutaneous leishmaniases caused by *L. tropica* and *L. braziliensis*, the levels of specific anti-sand fly saliva antibodies in humans positively correlated with the risk of *Leishmania* transmission [22,23]. In contrast, in foci of visceral leishmaniasis caused by *L. infantum*, levels of human anti-sand fly saliva antibodies positively correlated with anti-*Leishmania* DTH (delayed-type hypersensitivity) and thus with protection against potential infection [24,25]. So far, those studies have been performed only in humans. In canids, several studies showed presence of anti-sand fly saliva antibodies in sera from endemic areas in Brazil [8,26,27], however our study is the first describing the association with canine leishmaniasis.

Canine sera recognized more than eleven *P. perniciosus* antigenic bands by Western blot and the most intense reaction was often observed against a 42 kDa band. Mass spectrometry identified the 42 kDa band as a single protein belonging to the Yellow protein

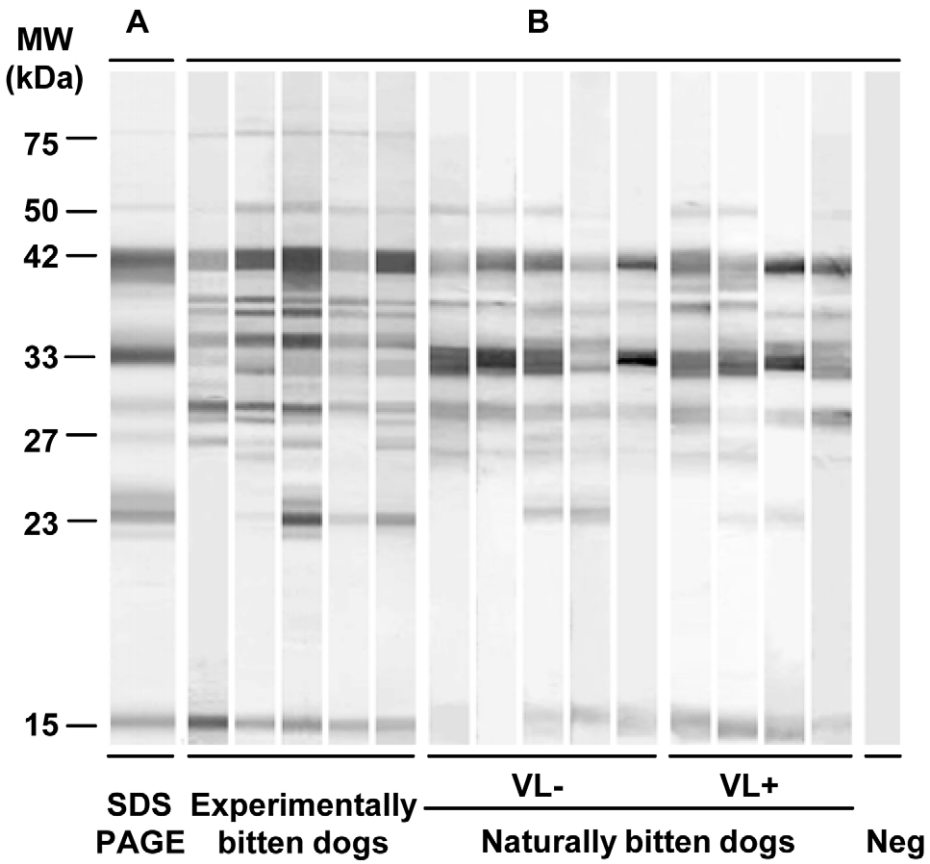

Figure 3. Anti-sand fly saliva antibody response in dogs experimentally and naturally bitten by *Phlebotomus perniciosus*. (A) Total protein profile, Commassie blue-stained SDS-PAGE gel after electrophoresis of *P. perniciosus* salivary gland homogenate. (B) Western blot of *P. perniciosus* salivary proteins recognized by sera of repeatedly bitten dogs. Western blot analysis was performed by sera of experimentally and naturally bitten dogs: *Leishmania infantum*-seronegative (VL−) and *L. infantum*-seropositive (VL+). Pre-immune serum of experimentally bitten dog was used as negative control (Neg).
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Table 3. *Phlebotomus perniciosus* salivary proteins recognized by sera of bitten dogs.

MW (kDa)	NCBI acc. number	Best match to NR protein database		
		Sequence name	E-value	Comments
75	DQ153102	29 kDa salivary protein (PpeSP08)	2.2e-6	unknown
50	DQ154099	41 kDa salivary protein (PpeSP32)	3.5e-9	endonuclease
42	DQ150622	43 kDa yellow-related salivary protein (PpeSP03B)	1.1e-68	yellow protein
40	DQ150621	42 kDa yellow-related salivary protein (PpeSP03)	4.5e-54	yellow protein
38	DQ192490	35.5 kDa salivary protein (PpeSP01)	5.6e-54	apyrase
38	DQ192491	35.3 kDa salivary protein (PpeSP01B)	0.035	apyrase
34	DQ153100	33 kDa salivary protein (PpeSP06)	2.2e-24	unknown
33	DQ192491	35.3 kDa salivary protein (PpeSP01B)	2.8e-72	apyrase
33	DQ153102	29 kDa salivary protein (PpeSP08)	0.0019	unknown
29	DQ153101	30 kDa antigen 5-related salivary protein (PpeSP07)	1.4e-12	Ag 5 protein
27	DQ153104	27 kDa D7-related salivary protein (PpeSP10)	0.0012	D7 protein
23	DQ150624	27 kDa D7-related salivary protein (PpeSP04B)	1.8e-16	D7 protein
23	DQ150623	24.5 kDa D7-related salivary protein (PpeSP04)	0.0069	D7 protein
14	DQ150620	14.8 kDa salivary protein (PpeSP02)	2.2e-13	SP15 like protein
14	DQ153105	13 kDa salivary protein (PpeSP11)	4.5e-15	SP15 like protein

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family (DQ150622). Previously, another Yellow protein of 47.3 kDa (AF132518) was reported as the major antigen recognized by sera of dogs bitten by *L. longipalpis* in the field [26]. The recombinant *L. longipalpis* Yellow proteins (rLJM11 and rLJM17) prepared in mammalian expression system kept their antigenicity and were successfully used to screen dog sera from Brazil [27], predicting similar features for Yellow protein of *P. perniciosus*. All canine sera tested recognized additional three major antigens of the 38, 33 and 29 kDa; the 38 and 33 kDa proteins are apyrases and the 29 kDa antigen represents the antigen 5-related protein family. These four antigens (42, 38, 33 and 29 kDa) are promising candidates as markers of sand fly exposure.

In conclusion, we confirmed that levels of antibodies against sand fly saliva positively correlate with the number of blood-fed sand flies and therefore, monitoring canine antibody response to specific sand fly salivary proteins may evaluate the need for, and effectiveness of, anti-vector campaigns. Moreover, this is the first study demonstrating relationship between the anti-sand fly saliva antibodies and the status of *L. infantum* infection in dogs. The levels of anti-*P. perniciosus* IgG2 in dogs naturally bitten by this sand fly species negatively correlate with the anti-*Leishmania* seropositivity. Thus, for dogs living in endemic area specific IgG2 response against saliva of the vector is suggested as a risk marker of *L. infantum* transmission.

Supporting Information

Figure S1 IFN-γ in the sera of *Leishmania infantum*-seropositive and -seronegative dogs naturally bitten by *Phlebotomus perniciosus* during the sand fly season. Concentrations of IFN-γ were measured by ELISA using the Quantikine canine IFN-γ immunoassay (R&D Systems) following the manufacturer's guidelines. Serum samples, standards and controls were added without any dilutions. Absorbance was measured at 450 nm using a Tecan Infinite M200 microplate reader (Schoeller). Data were transformed and assessed as described in manufacturer's instructions (R&D Systems).
(TIF)

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Author Contributions

Conceived and designed the experiments: PV NM DS DO. Performed the experiments: MV IR EK DS JD. Analyzed the data: MV IR PV. Contributed reagents/materials/analysis tools: PV MV IR NM DO. Wrote the paper: MV PV.

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Kinetics of Antibody Response in BALB/c and C57BL/6 Mice Bitten by *Phlebotomus papatasi*

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Abstract

Background: Phlebotomine sand flies are blood-sucking insects transmitting *Leishmania* parasites. In bitten hosts, sand fly saliva elicits specific immune response and the humoral immunity was shown to reflect the intensity of sand fly exposure. Thus, anti-saliva antibodies were suggested as the potential risk marker of *Leishmania* transmission. In this study, we examined the long-term kinetics and persistence of anti-*Phlebotomus papatasi* saliva antibody response in BALB/c and C57BL/6 mice. We also tested the reactivity of mice sera with *P. papatasi* salivary antigens and with the recombinant proteins.

Methodology/Principal Findings: Sera of BALB/c and C57BL/6 mice experimentally bitten by *Phlebotomus papatasi* were tested by ELISA for the presence of anti-saliva IgE, IgG and its subclasses. We detected a significant increase of specific IgG and IgG1 in both mice strains and IgG2b in BALB/c mice that positively correlated with the number of blood-fed *P. papatasi* females. Using western blot and mass spectrometry we identified the major *P. papatasi* antigens as Yellow-related proteins, D7-related proteins, antigen 5-related proteins and SP-15-like proteins. We therefore tested the reactivity of mice sera with four *P. papatasi* recombinant proteins coding for most of these potential antigens (PpSP44, PpSP42, PpSP30, and PpSP28). Each mouse serum reacted with at least one of the recombinant protein tested, although none of the recombinant proteins were recognized by all sera.

Conclusions: Our data confirmed the concept of using anti-sand fly saliva antibodies as a marker of sand fly exposure in *Phlebotomus papatasi*–mice model. As screening of specific antibodies is limited by the availability of salivary gland homogenate, utilization of recombinant proteins in such studies would be beneficial. Our present work demonstrates the feasibility of this implementation. A combination of recombinant salivary proteins is recommended for evaluation of intensity of sand fly exposure in endemic areas and for estimation of risk of *Leishmania* transmission.

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Introduction

Sand flies (Diptera: Phlebotominae) serve as vectors of leishmaniasis, a neglected disease with symptoms ranging from non-lethal cutaneous to life-threatening visceral form. The causative agents of the disease are protozoan parasites of the genus *Leishmania* which are transmitted to the hosts by the bites of infected sand fly females.

The percentage of infected flies in foci of leishmaniasis fluctuates and humans and animals are more frequently exposed to the bites of uninfected sand flies. Repeated exposure to sand fly saliva elicits anti-saliva antibodies that could be used as a marker of exposure to sand fly bites [1–5]. Moreover, the antibodies are sand fly species-specific. Therefore they can be utilized to differentiate between exposure to vector and non-vector species [1,4,6–9]. In several epidemiological studies, anti-sand fly saliva antibodies were already employed as a reliable tool to monitor exposure to sand fly bites, to evaluate the effectiveness of vector control programs, and in some instances to estimate the risk of *Leishmania* transmission [1,4,5,10–14].

In endemic areas sand fly population fluctuate seasonally [15], which may influence host anti-saliva antibody response. However, very little is known about the kinetics and persistence of anti-saliva antibodies in sera of hosts bitten by blood-feeding insects. Few data on antibody kinetics are available from mice, chicken and guinea pigs experimentally exposed to bites of *Triatoma infestans* [16–18], from humans bitten by mosquitoes [19–22] as well as from humans [4,23] and dogs [3,5,24] bitten by sand flies.

Screening for antibodies is, however, unsuitable for broader use in epidemiological studies until recombinant proteins could be employed instead of the crude salivary gland homogenate, which requires maintenance of sand fly colonies and laboratory

Author Summary

Leishmania major is the causative agent of zoonotic cutaneous leishmaniasis and *Phlebotomus papatasi* serve as the major vector. In endemic foci, rodents are the natural reservoirs of this disease. Thus, we studied anti-*P. papatasi* saliva antibody response in BALB/c and C57BL/6 mice that are commonly used as model organisms sensitive and resistant to cutaneous leishmaniasis, respectively. We followed the kinetics and persistence of specific antibody response in both mice strains and we characterized the main *P. papatasi* salivary antigens. We demonstrated that sand fly bites elicit production of specific IgG that reflect the intensity of sand fly exposure. In endemic areas, this could provide useful information about the effectiveness of anti-vector control programs. We also examined the reaction of mice sera with four *P. papatasi* recombinant proteins. Our data indicate that a combination of these proteins could be used instead of crude salivary gland homogenate for the monitoring of anti-sand fly saliva antibodies in natural hosts in endemic foci.

dissections of insects. So far, only recombinant salivary proteins from *Lutzomyia longipalpis* have been tested for reactivity with sera of naturally bitten humans, dogs, and foxes [8,9].

We studied mice antibody response to *P. papatasi*, the main vector of *Leishmania major*, and compared long-term kinetics and persistence of anti-saliva antibodies in BALB/c and C57BL/6 mice that are widely used as model organisms sensitive or resistant to *L. major* infection, respectively. Furthermore, we characterized and compared main *P. papatasi* salivary antigens recognized by sera of experimentally bitten BALB/c and C57BL/6 mice. The reactivity of mice sera was also tested with the four *P. papatasi* recombinant proteins; two Yellow-related proteins (PpSP44/AF335492 and PpSP42/AF335491) and two D7-related proteins (PpSP30/AF335489 and PpSP28/AF335488).

Methods

Ethical statement

BALB/c and C57BL/6 mice were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05; CZU 307/09) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic.

Sand flies and salivary gland dissection

A colony of *Phlebotomus papatasi* (originating from Turkey) was reared under standard conditions as described in [25]. Salivary glands were dissected from 4–6-day-old female sand flies, placed into 20 mM Tris buffer with 150 mM NaCl and stored at -20°C.

Experimental exposure

Twelve mice of BALB/c or C57BL/6 strains (6 weeks old) were divided into experimental and control groups of six mice each. Mice in the experimentally bitten groups were exposed individually to 30 *Phlebotomus papatasi* females (22 ± 0.6 (standard error) blood-fed females per mouse per exposure on BALB/C mice; 26 ± 0.7 (standard error) blood-fed females per mouse per exposure on C57BL/6 mice), once a week in a total of 5 exposures (weeks 1–5). Mice in the control groups remained without any exposure to sand flies. Animals in both groups were anaesthetized (ketamin 150 mg/kg and xylazin 15 mg/kg body weight, intraperitoneally). Blood samples were taken weekly from the tail vein of each mouse one day before exposure to sand flies from week 0 (pre-immune serum) to week 12 and than every other week till the end of the experiment (week 28 for BALB/c mice; week 27 for C57BL/6 mice). In total, mice were followed for 29 and 28 weeks, respectively. Two independent experiments were done for each mice strain.

To test the presence of memory cells, BALB/c mice were additionally exposed to *P. papatasi* bites (21 ± 0.5 (standard error) blood-fed females per mouse) in the week 27.

Preparation of recombinant proteins

Genes coding for *P. papatasi* salivary gland secreted proteins PpSP28 (AF335488), PpSP30 (AF335489), PpSP42 (AF335491) and PpSP44 (AF335492) were amplified from VR2001-TOPO vector [26] by PCR. Two specific restriction sites (*Nde* I and *Bam* HI) were incorporated into the PCR primers: PpSP28Fw (CATATGAAGTACCCTAGGAATGCCGAT), PpSP28Rev (GGATCCGTACGTTCTTGCGGATTGGTCATC), PpSP30Fw (CATATGCGATTTCCTAGGAATGGAGAC), PpSP30Rev (GGATCCGTATTTCCAAGATTCAATATCAAG), PpSP42Fw (CATATGAAAAGAGATGATGTTGGA), PpSP42Rev (GGATCCCCCTTGACACTTTTCTCC), PpSP44Fw (CATATGAAAAGAGACGATGTTGAA), and PpSP44Rev (GGATCCTTTAGGTTTTCTCACTTC). Afterwards, PCR products were ligated into *E. coli* pGEM-T Easy Vector (Promega) using TA cloning and the ligation products were transformed into *E. coli* competent cells TOP10 (Invitrogen). Vectors were replicated in bacteria and after that, genes restricted by *Nde* I and *Bam* HI enzymes and restricted *E. coli* pET-42 Expression Vectors (Novagen) were ligated. Ligation products were transformed into *E. coli* competent cells TOP10 (Invitrogen) again. Plasmids were isolated from the bacteria, and transformed into *E. coli* BL21 (DE3) gold (Agilent) for expression. *E. coli* lysates were prepared under denaturing conditions and His-tagged proteins were purified by FLPC on a Ni-NTA Superflow column with The QUIaxpressionist kit (Quiagen) according to manufacturers manual.

Detection of anti-*P. papatasi* saliva antibodies

Anti-*P. papatasi* saliva IgG antibodies and IgG subclasses were measured in sera of BALB/c and C57BL/6 mice using indirect ELISA. Microtiter plate wells were coated with *P. papatasi* salivary gland homogenate (SGH) made by three freeze-thaw cycles (about 60 ng of protein per well). To block free binding sites, washed wells were incubated with 6% low fat dry milk diluted in 20 mM phosphate-buffered saline with 0.05% Tween 20. Mice sera were diluted 1:200 in 2% low fat dry milk and incubated for 90 min at 37°C for specific IgG or overnight at 4°C for IgG subclasses. Secondary antibodies (goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgG3; Serotec) conjugated with horseradish peroxidase (HRP) were diluted and incubated at 37°C as described in Table S1. Orthophenylendiamine and H_2O_2 in McIlwein phosphate-citrate buffer (pH 5.5) were used as substrate solution. Absorbance was measured at 492 nm using an Infinite M200 microplate reader (Tecan). The cut-off value was determined as two standard errors of the mean of the absorbance of pre-immune serum. The

intensity of booster effect was measured by increased levels of specific antibodies in sera of bitten mice after the last sand fly exposure (comparing week 24 and 28).

Anti-*P. papatasi* IgE were measured in sera of BALB/c mice as described above with the following modifications. Microtiter plate wells were coated with *P. papatasi* SGH (about 300 ng of protein per well). To block the free binding sites, washed wells were incubated with 6% fetal calf serum. Mouse sera were diluted 1:100 in 2% fetal calf serum. Secondary antibody (rat anti-mouse IgE; BD PharMingen) was diluted and incubated as listed in Table S1.

Western blot analysis

Phlebotomus papatasi SGH (about 10 µg of protein per well) was separated on 10% SDS-PAGE gel under non-reducing conditions using the Mini-Protean III apparatus (BioRad). Salivary proteins were blotted onto a nitrocellulose membrane by Semi-Phor equipment (Hoefer Scientific Instruments) and cut into strips. The strips were then blocked with 5% low fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-Tw) and subsequently incubated with mice sera (week 28 for BALB/c mice; week 5 for C57BL/6 mice) diluted 1:200 for 1 hour. In the next step the strips were incubated for 1 hour with peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2b (Serotec) diluted in TBS-Tw as follows: IgG and IgG1 1:5000; IgG2b 1:2000 for BALB/c mice sera and IgG, IgG1 1:2000 for C57BL/6 mice sera. The chromogenic reaction was developed using a solution containing diaminobenzidine and H_2O_2.

Similar protocol was used for western blot analysis with *P. papatasi* recombinant proteins PpSP28, PpSP30, PpSP42, and PpSP44. Briefly, recombinant proteins were loaded on the 10% SDS-PAGE gel (3 µg protein per well) and separated under reducing conditions. BALB/c mice sera (week 28) were diluted 1:50 and secondary antibody (goat anti-mouse IgG from Serotec) was diluted 1:1000 in TBS-Tw.

Mass spectrometry

The proteins from the *P. papatasi* salivary glands used for mass spectrometric analysis were run on the same gel as salivary glands used for western blot analysis. Proteins were visualized by Coomassie Blue G-250 staining (Bio-Rad). The individual bands were cut and incubated with 10 mM dithiothreitol (DTT) and then treated with 55 mM iodoacetamid. Washed and dried bands were digested with trypsin (5 ng, Promega). Alpha-cyano-4-hydroxycinnamic acid was used as a matrix. Samples were measured using a 4800 Plus MALDI TOF/TOF analyzer (AB SCIEX). A peak list from MS spectra was generated by 4000 Series Explorer V 3.5.3 (AB SCIEX) without smoothing. Peaks with local signal to noise ratio greater than 5 were picked and searched by local Mascot v. 2.1 (Matrix Science) against a database of putative salivary protein sequences derived from GenBank. Database search criteria were as follows – enzyme: trypsin, taxonomy: *Phlebotomus*, fixed modification: carbamidomethylation, variable modification: methionine oxidation, peptide mass tolerance: 80 ppm, one missed cleavage allowed. Only hits that scored as significant ($p<0.05$) are included.

Statistical analysis

The data obtained by ELISA were subjected to GLM ANOVA and Tukey-Kramer Multiple Comparison procedure to analyze differences in kinetics of anti-*P. papatasi* saliva antibody response between experimentally bitten and control mice at all sampling points. The non-parametric Wilcoxon rank sum test for differences in medians was used for evaluation of booster effect, the comparison of antibody level between week 24 and 28. For correlation tests we used the non-parametric Spearman rank correlation matrix. For all tests statistical significance was regarded as a p-value less than 0.05. All statistical analyses were performed using NCSS 6.0.21 software.

Results

Kinetics of anti-*P. papatasi* saliva antibody response in BALB/c mice

To investigate the kinetics and persistence of anti-*P. papatasi* saliva antibody response, experimentally bitten and control mice were followed for 29 weeks. Mice exposed five times to bites of sand flies at one-week interval had significantly increased levels of specific IgG, IgG1, and IgG2b as compared to control group (Figure 1A, C, E). In contrast, specific IgG2a, IgG3, and IgE levels in sera of bitten mice were comparable to non-exposed controls with some differences only at the last data points (Figure S1). No anti-saliva antibodies were detected in any pre-immune sera tested.

In bitten mice, anti-*P. papatasi* saliva IgG and IgG1 levels increased significantly ($p<0.05$) after the fourth exposure (Figure 1A, C). IgG2b levels differed between experimental and control group from week 9 onward, with the exception of weeks 10 and 11 (Figure 1E). Anti-saliva IgG increased steadily till the end of the study, while specific IgG2b increased slowly until week 22 followed by a slight decrease at week 24. Anti-saliva IgG1 increased steadily and peaked at week 7 and persisted on this level until the end of the study.

To test the presence of putative memory cells to *P. papatasi* salivary proteins, BALB/c mice were additionally exposed to sand flies 22 weeks after the last exposure (week 27). One week after the booster (at week 28) anti-*P. papatasi* saliva antibodies increased significantly in IgG by 43%, in IgG1 by 80% and in IgG2b by 79% (Figure 1A, C, E).

Positive correlation was found between the number of blood-fed sand fly females during the individual immunization weeks (sum of the blood-fed females from the relevant week and the weeks before) and the corresponding levels of anti-*P. papatasi* IgG ($r = 0.62$, $p<0.0001$), IgG1 ($r = 0.74$, $p<0.0001$), and IgG2b ($r = 0.29$, $p<0.05$) (Figure 2A, C, E). Furthermore, positive correlation was detected between the total amount of blood-fed females and the levels of specific IgG ($r = 0.72$, $p<0.0001$) and IgG1 ($r = 0.8$, $p<0.0001$) after the fifth sand fly exposure (week 5).

Kinetics of anti-*P. papatasi* saliva antibody response in C57BL/6 mice

Experimentally bitten and control mice of C57BL/6 strain were followed in experiments lasting 28 weeks. Five exposures at one-week interval significantly increased levels of specific IgG and IgG1 in bitten mice (Figure 1B, D). In contrast, specific IgG2b, IgG2c, and IgG3 levels of bitten mice were comparable to controls. No anti-saliva antibodies were detected in any pre-immune sera tested.

Similarly to BALB/c mice, anti-*P. papatasi* IgG and IgG1 levels differed significantly between experimentally bitten and control C57BL/6 mice from week 4 onward (Figure 1B, D). Anti-saliva IgG gradually increased until week 8 and then with a slight fluctuation of antibody levels decreased until the end of the study. Specific IgG1 developed with similar kinetics to IgG, however, it peaked earlier (at week 6) and then slowly decreased till the end of

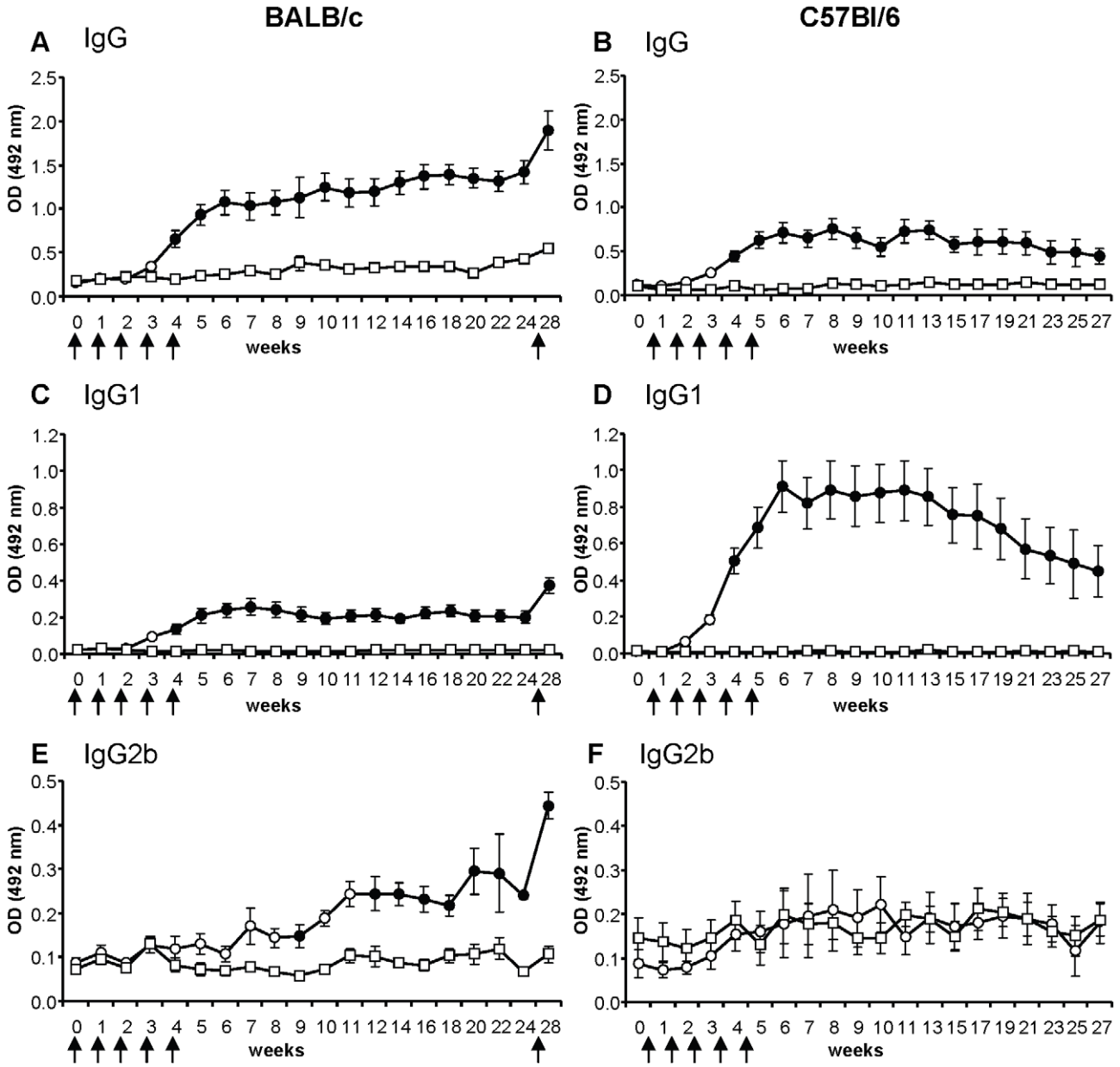

Figure 1. Anti-sand fly saliva antibody response in BALB/c and C57BL/6 mice bitten by *Phlebotomus papatasi*. BALB/c mice (A, C, E) and C57BL/6 mice (B, D, F) were divided into control (squares) and experimentally bitten groups (circles). Mice in the latter group were exposed to sand fly bites (arrows) in weeks 1–5 and additionally in the week 27 (only BALB/c mice). Levels of specific IgG (A, B); IgG1 (C, D); and IgG2b (E, F) were measured by ELISA at all sampling points. Full circles represent significant difference between control and bitten mice ($p<0.05$). Data are presented as the means ± standard errors of the means. Two independent studies were done. OD = optical density.
doi:10.1371/journal.pntd.0001719.g001

the study. Anti-saliva IgG2b, IgG2c, and IgG3 antibodies did not differ between the exposed and control group throughout the study (Figure 1F; Figure S1B, D) with the exception of week 21 for IgG3 subclass (Figure S1D).

We also detected a positive correlation between the number of blood-fed sand fly females during the individual immunization weeks (sum of the blood-fed females from the relevant week and the weeks before) and the corresponding levels of anti-*P. papatasi* IgG ($r = 0.80$, $p<0.0001$) and IgG1 ($r = 0.86$, $p<0.0001$) (Figure 2B, D). Moreover, positive correlation was detected between the total amount of blood-fed females and the levels of specific IgG ($r = 0.85$, $p<0.0001$), IgG1 ($r = 0.86$, $p<0.0001$), and IgG2c ($r = 0.5$, $p<0.05$) after the fifth sand fly exposure (week 5).

Identification and characterization of *P. papatasi* salivary antigens

Phlebotomus papatasi salivary antigens were studied using sera of experimentally bitten BALB/c and C57BL/6 mice. Only the antibody classes and subclasses shown to be produced in high titers by ELISA were tested in a western blot; specific anti-*P. papatasi* IgG and IgG1 in both mice strains and additionally specific IgG2b in BALB/c mice.

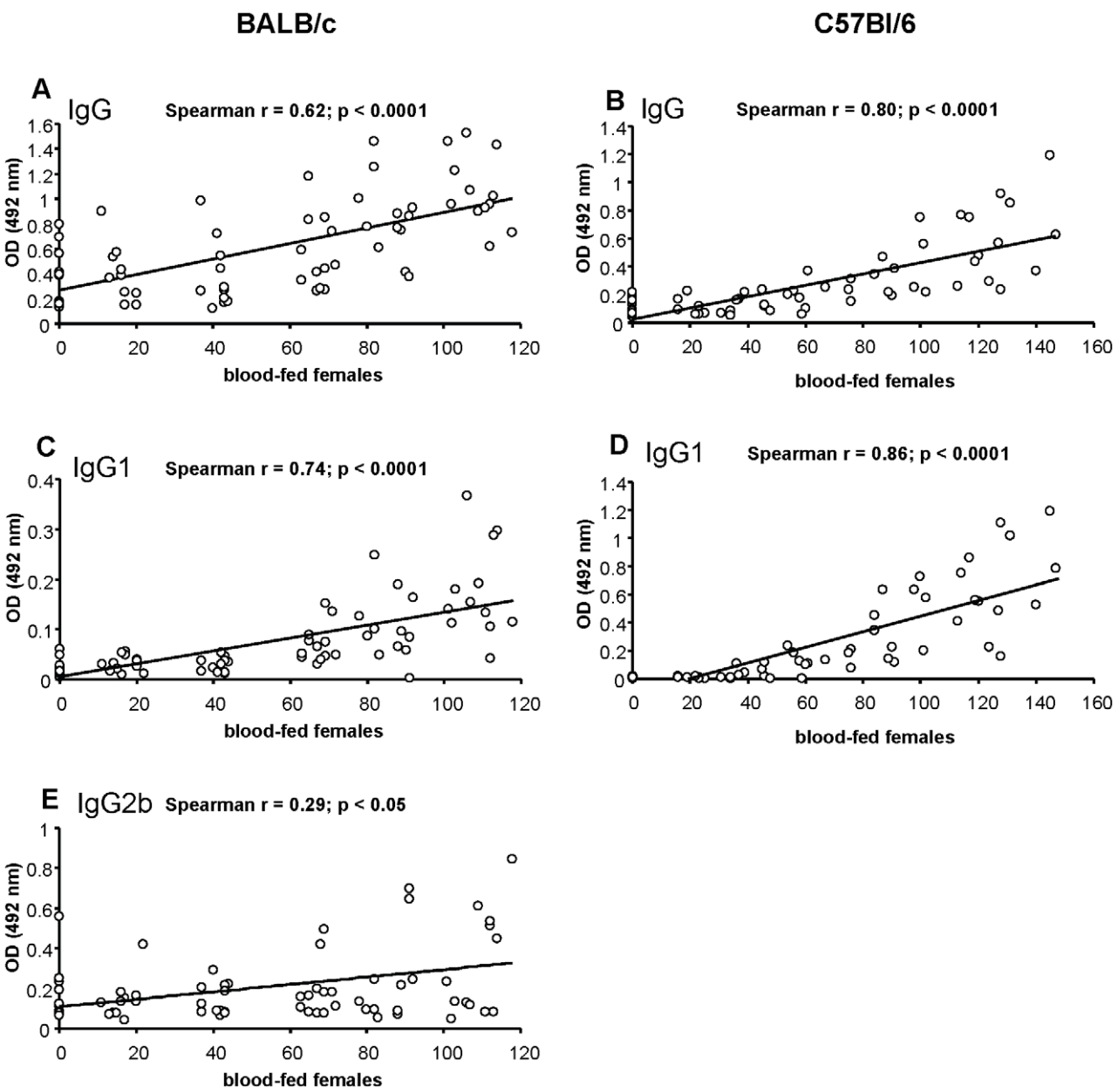

Figure 2. Correlation between the intensity of sand fly exposure and the anti-*Phlebotomus papatasi* saliva antibodies. The correlation between the number of blood-fed sand fly females and the levels of anti-saliva antibodies in experimentally bitten BALB/c (A, C, E) and C57BL/6 (B, D) mice was performed using Spearman Rank Correlation Matrix. Positive correlation was detected in specific IgG (A); IgG1 (C); and IgG2b (E) in BALB/c mice and in specific IgG (B); and IgG1 (D) in C57BL/6 mice. OD = optical density.
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BALB/c mice sera recognized up to 10 protein bands with approximate molecular weights of 70, 65, 51, 49, 47, 35, 31, 30, 23, and 15 kDa, the last three being the most intensively recognized by all BALB/c sera in all IgG subclasses tested. Sera of C57BL/6 mice reacted additionally with the 53 kDa protein but did not recognize the 49 and 47 kDa protein bands. The most intensive reaction in all C57BL/6 mice was detected with the 65, 53, and 30 kDa protein bands in IgG as well as in IgG1 (Figure 3). Comparison of two mice strains therefore revealed an interesting difference in recognition of four protein bands of 53, 51, 49, and 47 kDa. No reaction was detected with any pre-immune mice sera tested (Figure 3).

In BALB/c mice, the 51 kDa protein was recognized only by one out of 5 sera tested in IgG and IgG1, while in C57BL/6 mice, this protein band was recognized by all mice sera tested in IgG1 and by two out of five sera tested in IgG. Anti-*P. papatasi* IgG2b antibodies reacted consistently with the 65, 35, 31, 30, 23, and 15 kDa proteins (Figure 3).

In C57BL/6 mice, 70, 65, 53, 31, and 30 kDa proteins were recognized by all mice sera tested (IgG as well as IgG1), while the 51, 35, 23, and 15 kDa antigens were recognized by some sera only (Figure 3). Specific IgG1 of C57BL/6 mice predominantly recognized the 65, 53, 51, 31, and 30 kDa antigens (Figure 3).

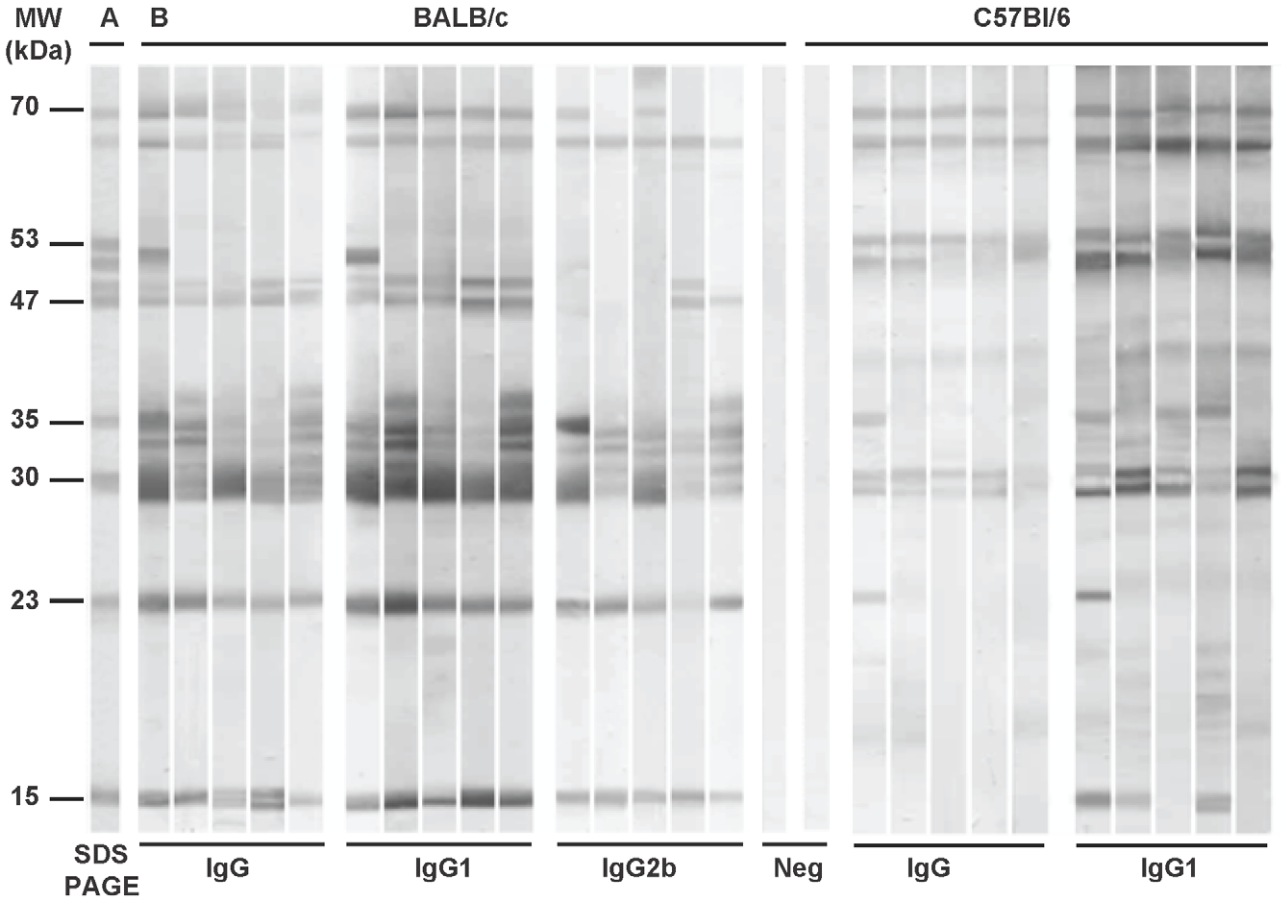

Figure 3. Anti-sand fly saliva antibody response in BALB/c and C57BL/6 mice experimentally bitten by *Phlebotomus papatasi*. (A) Total protein profile, Coomassie blue-stained SDS-PAGE gel with *P. papatasi* salivary gland homogenate. (B) Western blot of *P. papatasi* salivary proteins recognized by IgG, IgG1, or IgG2b from sera of *P. papatasi*-bitten BALB/c (week 28) and C57BL/6 mice (week 5). Pre-immune sera of BALB/c and C57BL/6 mice were used as negative controls (Neg).
doi:10.1371/journal.pntd.0001719.g003

Mass spectrometry analysis identified the salivary proteins with the same mobility in the SDS-PAGE as the proteins recognized by the sera of experimentally bitten mice as the Yellow-related proteins (GenBank acc. no. AF335492 and AF335491), apyrase (AF261768), D7-related proteins (AF335489; AF335488), antigen-5 protein (DQ205724), and proteins of the SP15 protein family (AY628879, AY628880; AF335486; AF335485) (Table 1).

Reactivity of anti-saliva antibodies with the *P. papatasi* recombinant proteins

The reactivity of PpSP44 (yellow related protein), PpSP42 (yellow related protein), PpSP30 (D7 related protein), and PpSP28 (D7 related protein) recombinant proteins was studied using sera from BALB/c mice exposed to *P. papatasi* bites and positive for anti-*P. papatasi* IgG antibodies. Sera of control mice did not recognize any of the recombinant proteins tested. The most intensive reaction was detected with the PpSP30, although, this protein was not recognized by all sera tested (4 out of 5). Three out of five mice sera reacted with the PpSP42 and PpSP44 recombinant proteins and very weak reaction was detected with the PpSP28 recombinant protein in two out of five mice sera (Figure 4).

Discussion

This study describes in detail long-term kinetics and persistence of anti-*P. papatasi* saliva antibodies in sand fly-exposed BALB/c and C57BL/6 mice strains that are widely used as model organisms sensitive or resistant to *Leishmania* infection, respectively (e.g. [27,28]).

Four IgG subtypes have been described in mice: IgG1, IgG2a, IgG2b, and IgG3. Additionally, certain strains such as C57BL/6 produce the IgG2c subclass instead of IgG2a [29]. The nomenclature of murine IgG subtypes does not correlate with the subtypes of human or canine IgG. The most abundant subclass is IgG1; it binds to Fc-receptors of mast cells and basophils, and it mediates the immediate hypersensitivity reactions. Both IgG1 and IgG2a activate the complement cascade via the alternative pathway, whereas IgG2b employs the classical pathway of complement activation [30]. Moreover, production of IgG1 is the marker of Th2 profile of immune response in mice, while IgG2a predicts Th1 type of immune response in these animals [31].

We showed that repeated exposure to sand fly bites elicits increased levels of anti-saliva IgG and IgG1 in both BALB/c and C57BL/6 strains, and additionally IgG2b in BALB/c mice. In comparison, higher levels of specific IgG were detected in BALB/c mice. This finding complies well with the fact that BALB/c mice mostly respond to repeating antigens by Th2 humoral immune response while C57BL/6 mice produce mainly Th1 cellular response [30]. It seems that *P. papatasi* saliva elicits mainly production of specific IgG1 subclass, which suggests the polariza-

Table 1. *Phlebotomus papatasi* salivary proteins recognized by sera of bitten mice.

MW	NCBI acc.number	Best match to NR protein database		
(kDa)		Sequence name	E-value	Protein family
70	N.D.	N.D.	N.D.	N.D.
65	N.D.	N.D.	N.D.	N.D.
53	N.D.	N.D.	N.D.	N.D.
51	AF335492	44 kDa yellow-related salivary protein (PpSP44)	0.00E+00	yellow-related
49	N.D.	N.D.	N.D.	N.D.
47	AF335491	42 kDa yellow-related salivary protein (PpSP42)	0.00E+00	yellow-related
35	AF261768	salivary apyrase (PpSP36)	0.00E+00	apyrase
31	AF335490	32 kDa salivary protein (PpSP32)	1.10E−05	PpSP32-like
30	AF335489	30 kDa D7-related salivary protein (PpSP30)	4.50E−12	D7-related
30	DQ205724	29 kDa antigen 5-related salivary protein	7.10E−05	antigen 5-related
23	AF335488	28 kDa D7-related salivary protein (PpSP28)	0.00E+00	D7-related
15	AY628879	SP-15 protein	1.80E−05	PpSP15-like
15	AY628880	SP-15 protein	3.50E−07	PpSP15-like
15	AF335486	14 kDa salivary protein (PpSP14)	0.00E+00	PpSP15-like
15	AF335485	12 kDa salivary protein (PpSP12)	0.00E+00	PpSP15-like

N.D. – not determined.

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tion to the Th2 type of immune response in bitten mice regardless of the strain. The production of anti-sand fly saliva IgG1 was previously described in BALB/c mice repeatedly bitten by *Lutzomyia longipalpis*, but they did not observe any production of neither IgG2a nor IgG2b [32]. As the composition of sand fly saliva varies in different sand fly species [33] and the sand fly saliva compounds elicit different profile of specific antibody response [34], this could be the feasible explanation for the production of different antibody subclasses in mice bitten by different sand fly species. To our knowledge, there are no data available about the anti-sand fly saliva antibody subclasses elicited by sand fly feeding in the C57BL/6 mice. In Swiss Webster mice immunization by *P. ariasi* saliva produced also predominantly IgG1 antibodies [34]. Production of specific IgG2b in BALB/c mice compared to the

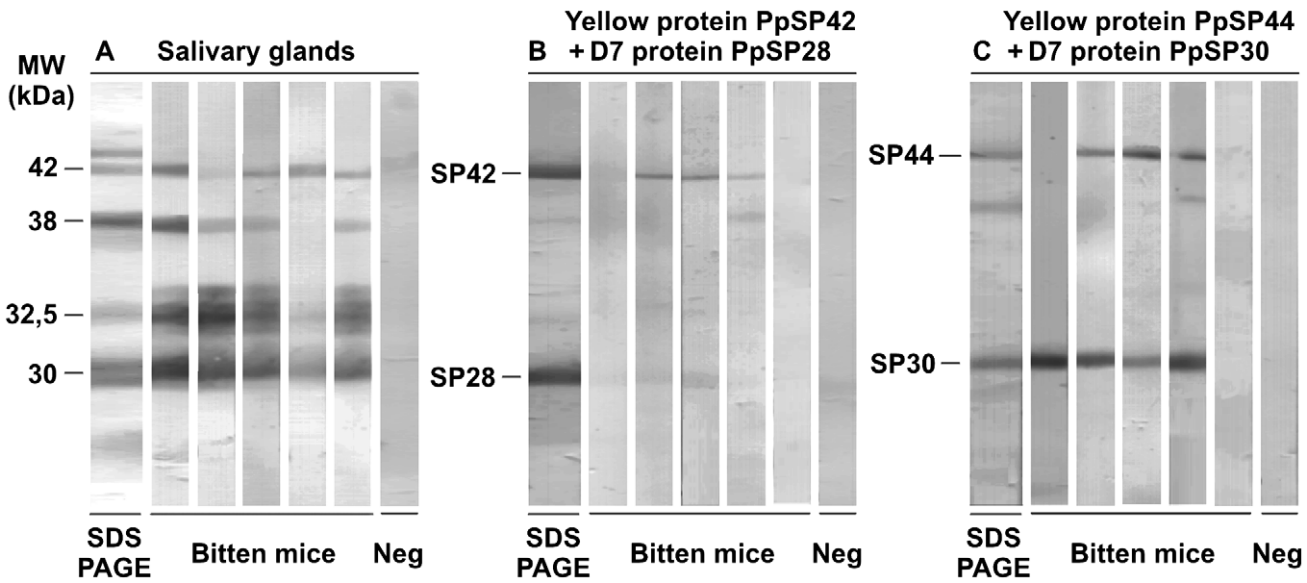

Figure 4. Reactivity of anti-*Phlebotomus papatasi* saliva IgG with *P. papatasi* recombinant proteins. Sera from BALB/c mice experimentally bitten by *P. papatasi* (Bitten mice) that were positive for anti-*P. papatasi* IgG (OD>cut-off: 0.19) were tested by Western blot analysis against (A) *P. papatasi* salivary gland homogenate, (B) a combination of bacterially-expressed PpSP42 and PpSP28 (Yellow-related protein AF335491 and D7-related protein AF335488, respectively), and (C) a combination of bacterially-expressed PpSP44 and PpSP30 (Yellow-related protein AF335492 and D7-related protein AF335489, respectively). The SDS-PAGE protein profiles of the *P. papatasi* salivary gland homogenate as well as the recombinant proteins were blotted and stained by Amido Black. Pre-immune sera of BALB/c mice were used as the controls (Neg).

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absence of this antibody subclass in C57BL/6 mice may be the result of different cytokine responses in both mice strains against sand fly saliva. The switch to IgG2b subclass is initialized by production of TGF-β [35], a suppressive cytokine that blocks the activation of lymphocytes and monocytes derived phagocytes. This could positively contribute to the susceptibility of BALB/c mice to *Leishmania* parasites.

Importantly, positive correlation was found in both mice strains between the intensity of sand fly exposure and the levels of specific antibodies in aforementioned subclasses. Our results correspond well to previously published data showing that the antibody response in dogs [3,5] as well as in humans [4] reflected the intensity and the time-course of sand fly exposure.

We found that sand fly exposure did not affect the production of IgG2a and IgG3 in BALB/c mice, and IgG2b, IgG2c, and IgG3 in C57BL/6 mice. Neither did the levels of specific IgE differ significantly between non-exposed and exposed groups of mice, and the IgE kinetics showed high variation during the study. Similarly, high fluctuation in specific IgE response was detected in humans [11,23] and dogs [3] bitten by *Lutzomyia longipalpis* in the field as well as under laboratory conditions. While some of the individuals and animals presented high levels of specific IgE, others did not mount specific IgE response at all [3,11,23].

To mimic the situation commonly occurring in endemic foci of leishmaniases, where sand fly-free periods last up to 6 months [15], BALB/c mice were exposed to *P. papatasi* bites again 23 weeks after the last sand fly exposure. This single sand fly exposure elicited statistically significant increase of anti-*P. papatasi* IgG, IgG1, IgG2b which suggests the persistence of memory cells generated during the previous round of exposures. This could be related to the ''previous sand fly season'' in the field. Furthermore, in both mice strains, the differences between non-exposed and exposed groups of mice in production of specific IgG1 and IgG2b were detectable from week four or nine, respectively, until the end of the study. Similarly, the levels of specific IgG, IgG1, and IgG2 in sera of dogs exposed to *L. longipalpis* or *P. perniciosus* bites differed significantly from pre-immune sera for more than 14 weeks after the last sand fly exposure [3,5]. In individuals repeatedly bitten by *P. argentipes*, elevated levels of specific antibodies persisted after the 30-day sand fly-free period, although anti-saliva antibodies significantly decreased throughout this time [4]. Thus, regardless the host-sand fly combination, anti-sand fly saliva antibodies can persist in sera of repeatedly bitten hosts until the next sand fly season.

We also characterized the reactivity of mice sera with *P. papatasi* salivary proteins as well as with selected recombinant proteins. Mice sera of BALB/c and C57BL/6 strains reacted with up to eleven *P. papatasi* antigenic protein bands. The 30 kDa protein band recognized by both mice strains was identified by mass spectrometry as a mixture of a D7-related (AF335489) and an antigen 5-related (DQ205724) protein. The other proteins which were intensively recognized either by BALB/c (47, 23, and 15 kDa proteins) or by C57BL/6 mice (65, 53, and 51 kDa proteins) were determined as members of the Yellow-related protein family (51 kDa - AF335492, 47 kDa - AF335491), D7-related protein family (23 kDa – AF335488), and SP-15 protein family (15 kDa – AY628879, AY628880, AF335486, AF335485). The 70, 65, 53, and 49 kDa bands were not identified by mass spectrometry. Our results correspond to previously published data, where the human and BALB/c mice IgG antibodies recognized preferentially the *P. papatasi* 30 kDa protein band [1,14].

To our knowledge, the only study describing the reactivity of specific IgG subclasses with *P. papatasi* antigens was performed on humans [14]. In accordance with our results, the 30 kDa D7-related protein was also found to be the most immunogenic antigen in all human antibody subclasses tested [14]. Taken together, our data complies well with previously published studies, where Yellow-related proteins, D7-related proteins, as well as SP-15 proteins from *P. papatasi* saliva were identified as potent antigens for mice and humans [1,14].

Sera of BALB/c mice experimentally bitten by *P. papatasi* were tested also with four bacterially expressed recombinant proteins belonging to two salivary protein families: Yellow-related proteins (PpSP44/AF335492 and PpSP42/AF335491) and D7-related proteins (PpSP30/AF335489 and PpSP28/AF335488). Within the salivary gland homogenate, sera reacted with proteins identified as PpSP42, PpSP30, and PpSP28 proteins, but no reaction was detected with PpSP44. In contrast, PpSP30 and PpSP44 recombinant proteins were strongly recognized and PpSP42 gave a weak reaction. Reaction of anti-saliva IgG with recombinant proteins may, however, differ between mouse strains. For example, the C57BL/6 mice reacted predominantly with PpSP42 and PpSP28 recombinant proteins (data not shown). Although none of the recombinant proteins were recognized by all sera. Each mouse serum tested reacted with at least one of the recombinant proteins.

Our data suggest that recombinant proteins could be used as markers of sand fly exposure instead of crude salivary gland homogenates, ideally as a mixture of several different proteins to cope with various host species and individual reactivity of each serum sample. In sand flies this concept has been demonstrated using *Lutzomyia longipalpis* recombinant proteins; the reactivity of anti-*L. longipalpis* seropositive human sera with the salivary gland sonicate was comparable to the reaction with the combination of the two *L. longipalpis* recombinant Yellow-related proteins (LJM11/AY445935 and LJM17/AF132518) [8].

In conclusion, we detected a significant increase of specific IgG and IgG1 in exposed mice of both strains, and of IgG2b in exposed BALB/c mice. The other IgG subclasses were comparable to controls. Specific IgG response was shown to reflect the intensity of sand fly exposure and furthermore, anti-*P. papatasi* saliva antibody response persisted in mice for more than 5 months. Thus, in endemic areas the antibodies could persist till the following sand fly season. The 30 kDa band recognized by sera of experimentally bitten BALB/c as well as C57BL/6 mice was identified as a mixture of D7-related and antigen 5-related proteins. Moreover, the reactivity of mice sera with PpSP44, PpSP42, PpSP30, and PpSP28 recombinant proteins suggested that their combination could substitute the salivary gland homogenate. Taken together, the kinetics, persistence and the individual variability of anti-sand fly saliva antibody response are important aspects to consider in further experiments, where anti-saliva antibodies are used as the markers of sand fly exposure.

Supporting Information

Figure S1 Anti-sand fly saliva antibody response in BALB/c and C57BL/6 mice bitten by *Phlebotomus papatasi*. BALB/c mice and C57BL/6 mice were divided into control (squares) and experimentally bitten groups (circles). Mice in the latter group were exposed to sand fly bites (arrows) (30 *P. papatasi* females per week) in weeks 1–5 and additionally in the week 27 (only BALB/c mice). Anti-*P. papatasi* saliva antibodies - IgG2a (A), IgG3 (C), and IgE (E) in BALB/c mice and IgG2c (B) and IgG3 (D) in C57BL/6 mice - were measured using ELISA as described in Methods. Data are presented as the means ± standard errors of the means. Two independent studies were done. OD = optical density.
(TIF)

Table S1 Dilution and incubation time of secondary antibodies.
(DOC)

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Author Contributions

Conceived and designed the experiments: IR JH PV. Performed the experiments: MV JH LP LZ JD. Analyzed the data: MV IR JH LP PV. Contributed reagents/materials/analysis tools: MV IR JGV PV. Wrote the paper: MV IR PV.

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Recombinant Antigens from *Phlebotomus perniciosus* Saliva as Markers of Canine Exposure to Visceral Leishmaniases Vector

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Abstract

Background: *Phlebotomus perniciosus* is the main vector in the western Mediterranean area of the protozoan parasite *Leishmania infantum*, the causative agent of canine and human visceral leishmaniases. Infected dogs serve as a reservoir of the disease, and therefore measuring the exposure of dogs to sand fly bites is important for estimating the risk of *L. infantum* transmission. In bitten hosts, sand fly saliva elicits a specific antibody response that reflects the intensity of sand fly exposure. As screening of specific anti-saliva antibodies is limited by the availability of salivary gland homogenates, utilization of recombinant salivary proteins is a promising alternative. In this manuscript we show for the first time the use of recombinant salivary proteins as a functional tool for detecting *P. perniciosus* bites in dogs.

Methodology/Principal Findings: The reactivity of six bacterially-expressed recombinant salivary proteins of *P. perniciosus*, yellow-related protein rSP03B, apyrases rSP01B and rSP01, antigen 5-related rSP07, ParSP25-like protein rSP08 and D7-related protein rSP04, were tested with sera of mice and dogs experimentally bitten by this sand fly using immunoblots and ELISA. In the immunoblots, both mice and canine sera gave positive reactions with yellow-related protein, both apyrases and ParSP25-like protein. A similar reaction for recombinant salivary proteins was observed by ELISA, with the reactivity of yellow-related protein and apyrases significantly correlated with the antibody response of mice and dogs against the whole salivary gland homogenate.

Conclusions/Significance: Three recombinant salivary antigens of *P. perniciosus*, yellow-related protein rSP03B and the apyrases rSP01B and rSP01, were identified as the best candidates for evaluating the exposure of mice and dogs to *P. perniciosus* bites. Utilization of these proteins, or their combination, would be beneficial for screening canine sera in endemic areas of visceral leishmaniases for vector exposure and for estimating the risk of *L. infantum* transmission in dogs.

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Introduction

Canine leishmaniasis (CanL) is a systemic disease with variable clinical symptoms. Its causative agent, the protozoan parasite *Leishmania infantum*, is transmitted by phlebotomine sand flies (Diptera: *Phlebotominae*). CanL occurs frequently around the Mediterranean Basin and in many countries in Latin America, where the prevalence of infection often exceeds 25%. Dogs with inapparent infections often play a role in the circulation of the parasite, as they are able to infect sand flies (reviewed in [1]). New cases of autochthonous leishmaniasis caused by *L. infantum* have been occurring in various countries, suggesting an expansion of CanL towards new biotopes at higher latitudes and higher altitudes (reviewed in [2,3,4]). Importantly, CanL is not just a veterinary problem; infected dogs serve as a reservoir host of human visceral leishmaniasis and there is a correlation between the prevalence of leishmaniasis in the canine population and the human disease in many countries [1].

Two sand fly genera are involved in *L. infantum* transmission, *Lutzomyia* in the New World and *Phlebotomus* in the Old World. Seven species of the genus *Phlebotomus*, subgenus *Larroussius*, are proven or probable vectors of CanL in different places around the Mediterranean Basin [5]. Of these, *Phlebotomus perniciosus* has the widest distribution, with ranges in both the southern and northern parts of the Mediterranean, from Morocco and Portugal in the west to Italy in the east and Germany in the north [5].

Measuring the exposure of dogs to sand fly bites is important for estimating the risk of *L. infantum* transmission. Recently, it has been

Author Summary

The protozoan parasite *Leishmania infantum* is a causative agent of zoonotic visceral leishmaniasis, an important and potentially fatal human disease. The main reservoir hosts of this *Leishmania* species are dogs, and the only proven vectors are phlebotominae sand flies, *Phlebotomus perniciosus* being considered the major vector in the western Mediterranean area. During feeding on the host, sand flies spit saliva into the host skin; hosts develop a specific antibody response directed against sand fly salivary proteins and levels of these antibodies reflect the intensity of sand fly exposure. As the availability of salivary gland homogenate is limited, recombinant salivary proteins have been suggested as antigens suitable for measuring specific antibody levels. In the present work, we expressed six of the most-antigenic salivary proteins, and studied the mice and canine humoral immune responses to these recombinant proteins. We demonstrated that three proteins, a yellow-related protein and two apyrases, are suitable antigens for measuring anti-*P. perniciosus* antibody levels and estimating the host exposure to this sand fly species.

demonstrated that experimental exposure of dogs to *L. longipalpis* or *P. perniciosus* bites elicits the production of specific anti-saliva IgG (measured by ELISA with whole SGH) that positively correlates with the number of sand fly bites [6,7]. The elicitation of IgG antibody levels persists for at least five weeks after last exposure of dogs to *P. perniciosus* [7] or even nineteen weeks after dogs are exposed to *L. longipalpis* [6]. Therefore, monitoring canine IgG levels specific for sand fly saliva could indicate their exposure to sand fly bites. Such a monitoring technique would be useful for evaluating the need for, and effectiveness of, anti-vector campaigns [6,8]. However, obtaining sufficient native antigens through sand fly dissections for the upscaled production of such antigens necessary for ELISA is not feasible; thus there is a need to replace native antigens in the ELISAs by recombinant antigens. To date, recombinant salivary proteins from *Phlebotomus papatasi* have been used to detect the antibody response in mice experimentally exposed to this sand fly species [9], and *L. longipalpis* recombinant antigens have been tested for reactivity with the sera of naturally bitten humans, dogs and foxes [10,11].

Recent studies [7,12] have shown that the sera of dogs bitten by *P. perniciosus* recognize up to thirteen antigens in the salivary gland homogenate (SGH) of this species. The most intense reaction has repeatedly been observed against the 43 kDa yellow-related protein PpeSP03B (referred to further in the text as yellow protein rSP03B), the 35.3 kDa PpeSP01B and 35.5 kDa PpeSP01 kDa apyrases (apyrase rSP01B and rSP01, respectively), the 30 kDa antigen 5-related protein PpeSP07 (antigen 5 rSP07), the 29 kDa ParSP25-like protein PpeSP08 (ParSP25 protein rSP08), and the 24.5 kDa D7-related salivary protein PpeSP04 (D7 protein rSP04). The recombinant forms of these six proteins were therefore chosen in the present work as the most promising candidates for markers of *P. perniciosus* exposure.

Materials and Methods

1. Ethics statement

SKH1-hr mice were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05; CZU 307/09) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic.

2. Sand flies and salivary gland homogenate

Two *Phlebotomus* (*Larroussius*) *perniciosus* sand fly colonies originating from Spain – from Murcia and from Madrid – were used, and maintained under standard conditions described in [13,14].

Phlebotomus perniciosus salivary glands for immunoblots and ELISA assays were dissected from 3 to 5-day-old females, and pools of 20 salivary glands in 20 µl of Tris-NaCl buffer (20 mM Tris, 150 mM NaCl, pH 7.6) were stored at −80°C. Salivary gland homogenate (SGH) was obtained by disruption of the glands with repeated freezing and thawing cycles. Salivary glands used for isolation of mRNA were dissected from 1-day-old female sand flies, and were stored in groups of 20 salivary glands in 20 µl of RNAlater (Qiagen) and kept at −80°C until use.

3. Mice and canine sera

Hyperimmune mice sera were obtained by repeated exposure of three SKH1hr mice to bites of uninfected *P. perniciosus* females; three other mice of the same strain served as non-exposed controls. Canine sera from dogs (beagles) experimentally exposed to *P. perniciosus* bites were selected from those previously used for studies on SGH [7]. Sera were chosen to cover a wide range of anti-saliva antibody levels: six originated from highly exposed dogs (bitten by 150–190 sand fly females once a week), six from dogs with lower exposure (20–70 females once a week) and six from unexposed dogs (before exposure). The sera of exposed dogs were collected seven days after the fourth exposure.

4. Recombinant salivary proteins

Six bacterially-expressed recombinant antigens expressed in nine forms were studied: yellow protein rSP03B (KF257369), two apyrases - rSP01B (KF257364, KF257366, KF178455) and rSP01 (KF257365, KF257367), antigen 5 protein rSP07 (KF257368), ParSP25 protein rSP08 (KF178457) and D7 protein rSP04 (KF178456). Coding sequences of SP01 and SP01B (referred to further in the text as rSP01B/1 in pET42b and rSP01/1 in pET42b, respectively), SP07 and SP03B were obtained from salivary glands of *P. perniciosus* (Murcia colony). After dissection into RNAlater, mRNA was isolated by a Roche High Pure RNA Tissue Kit and transcripted to cDNA by a Roche High Fidelity cDNA Synthesis Kit using polyA primers. The second cDNA strands of SP01B and SP01 were amplified by PCR using specific primers that were synthesized according to the sequences of the mature protein (without signal peptide). These sequences have been published with the following accession numbers [15]: rSP01B/1 - DQ192491 and SP01/1 - DQ192490. Afterwards, we followed the procedure that was described in [9] – briefly, genes were expressed in pET42b (Novagen) with a His tag containing 8 histidines – the *E.coli* BL21 (DE3) expression system. In parallel, single-stranded cDNA transcripted from salivary glands as described above was sent to Apronex s.r.o. (Prague) for preparation of rSP01, rSP01B, rSP03B and rSP07 proteins in the recombinant form according to sequences published in the cDNA library [15] - rSP03B (DQ150622), rSP01B/2 (DQ192491), rSP01/2 (DQ192490), rSP07 (DQ153101). All four proteins were expressed in the *E.coli* BL21 (DE3) expression system; apyrases rSP01B/2 and rSP01/2 were expressed in the pET42b vector

(Novagen) with 2 His tags - the first containing 6 histidines and the second 8 histidines, and a 1.4 kDa adaptor, while yellow protein rSP03B and antigen 5 protein rSP07 were expressed in the pET28b vector (Novagen) with 1 His tag containing 6 histidines. All proteins were isolated under denaturing conditions with 8M urea.

In addition, salivary coding sequences of SP01B, SP04 and SP08 were obtained from a cDNA library constructed from the salivary glands of *P. perniciosus* from Madrid – GenBank accession numbers are: SP01B - HE974345.1, SP04 - HE980444.1, SP08 - HE974347.1; in contrast to the other proteins tested, they contain signal sequences (thus they seem to be about 3 kDa heavier on immunoblots). Recombinant proteins rSP01B and rSP04 were expressed in the pQE31 vector (Qiagen) with a His tag containing 6 histidines in *E. coli* M15 cells, and purified under denaturing conditions with 8M urea. Protein rSP08 was cloned into the pGEX4T3 vector (Amersham Biotech), expressed in *E. coli* Arctic Express cells (Agilent), and purified by polyacrylamide gel extraction in PBS. As rSP08 was expressed as a fusion protein with gluthathione S-transferase (GST), the latter was also obtained and used as a control in immunoblots and ELISA. In all these cases, protein refolding was performed using dialysis against PBS using SnakeSkin Dialysis Tubing (10 kDa MWCO, Thermo Scientific Goettiengen, Germany).

The concentration of all proteins was quantified by the Lowry method (Bio-Rad) following the manufacturer's protocol.

5. Immunoblots

The immunogenicity of the recombinant proteins was tested by the immunoblot technique. Recombinant salivary proteins were separated by SDS PAGE on 12% polyacrylamide gels under non-reducing conditions using a Mini-protean apparatus (Bio-Rad). Protein concentration was optimized using preliminary experiments; the list of proteins and their quantity per well of the 5-well comb are given in Table 1. Proteins were either stained with Coomassie Blue (Invitrogen) or transferred from the gel to nitrocellulose membranes using an iBLOT dry system (Invitrogen).

Membranes were cut into strips (area corresponding to one well was cut into 5 strips), blocked for 1–2 hours with 5% milk in Tris buffer with 0.05% Tween (Tris-Tw) and then incubated for 1 hour with either mice or canine sera diluted in Tris-Tw. Mice sera were diluted 1:200 for denatured proteins rSP01B and rSP01 in pET42b and 1:100 in the case of other proteins; canine sera were used at a dilution of 1:50. After washing in Tris-Tw, the strips were incubated with peroxidase conjugated anti-mouse IgG (1:1000, AbD Serotec) or anti-dog IgG antibodies (1:1000, Bethyl) and reacting protein bands were visualized using the substrate solution with diaminobenzidine.

6. ELISA

The ELISA test described elsewhere [7,16] was modified as follows. Covalink plates (Nunc) were coated with 100 µl of either salivary gland homogenate (40 ng of protein per well, corresponds to 1/5 of a salivary gland) or recombinant salivary protein, both in 0.1 M carbonate-bicarbonate buffer (pH 9.5), overnight at 4°C. The optimal concentrations of the recombinant proteins are given in Table 1. After washing in PBS with 0.05% Tween (PBS-Tw), plates were blocked with 6% milk in PBS-Tw for 1–2 h at 37°C.

Mice and canine sera were diluted in 2% milk PBS-Tw. Dilution of mice sera 1:1600 was optimal for rSP03B, 1:400 for apyrases in pET42b with 1 His tag, and 1:200 for the other proteins tested. All canine sera were used at a dilution of 1:50. Sera were incubated for 90 min at 37°C. Following washing cycles, plates were incubated with peroxidase-conjugated anti-mouse IgG (AbD Serotec) or anti-dog IgG (Bethyl) and the color reaction was developed in the substrate solution with orthophenylendiamine. Absorbance values (OD) were recorded at 492 nm using a Multiscan RC ELISA reader (Labsystems).

7. Statistical analysis

The non-parametric Spearman test was used to assess correlations between total anti-SGH and anti-recombinants antibody levels in GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA). Statistical significance was considered when the p-value was <0.05.

8. Accession numbers

The sequences of apyrases rSP01B/1 (in pET42b), rSP01B/2 (in pET42b), rSP01/1 (in pET42b) and rSP01/2 (in pET42b), yellow protein rSP03B (in pET28b) and antigen 5 protein rSP07 (in pET28b) were based on sequences from a published cDNA library of *P. perniciosus* [15]: rSP01B - DQ192491, rSP01 - DQ192490, rSP03B - DQ150622) and rSP07 - DQ153101. The other sequences were published directly in GenBank: rSP01B (in pQE31) - HE974345.1, rSP04 (in pQE31) - HE980444.1, rSP08 (pGEX4T3) - HE974347.1. The expressed sequences were published with the following GenBank accession numbers: yellow protein rSP03B (KF257369), two apyrases - rSP01B (KF257364, KF257366, KF178455) and rSP01 (KF257365, KF257367), antigen 5 protein rSP07 (KF257368), ParSP25 protein rSP08 (KF178457) and D7 protein rSP04 (KF178456).

Table 1. Concentrations of recombinant proteins used for immunoblots (µg per well) and ELISA (µg/ml) with mice and canine sera.

	Protein	Immunoblot	ELISA mouse	ELISA dog
Denatured	Apyrase rSP01B/1 pET42b	3	6	9
	Apyrase rSP01/1 pET42b	4	6	6
	Apyrase rSP01B/2 pET42b	9	6	9
	Apyrase rSP01/2 pET42b	6	3	3
	Yellow protein rSP03B pET28b	4	3	3
	Antigen 5-related protein rSP07 pET28b	9	9	9
Refolded	Apyrase rSP01B pqE31	9	35	35
	D7-related protein rSP04 pqE31	9	18	18
	ParSP25-like protein rSP08 pGEX4T3	13	3	13

doi:10.1371/journal.pntd.0002597.t001

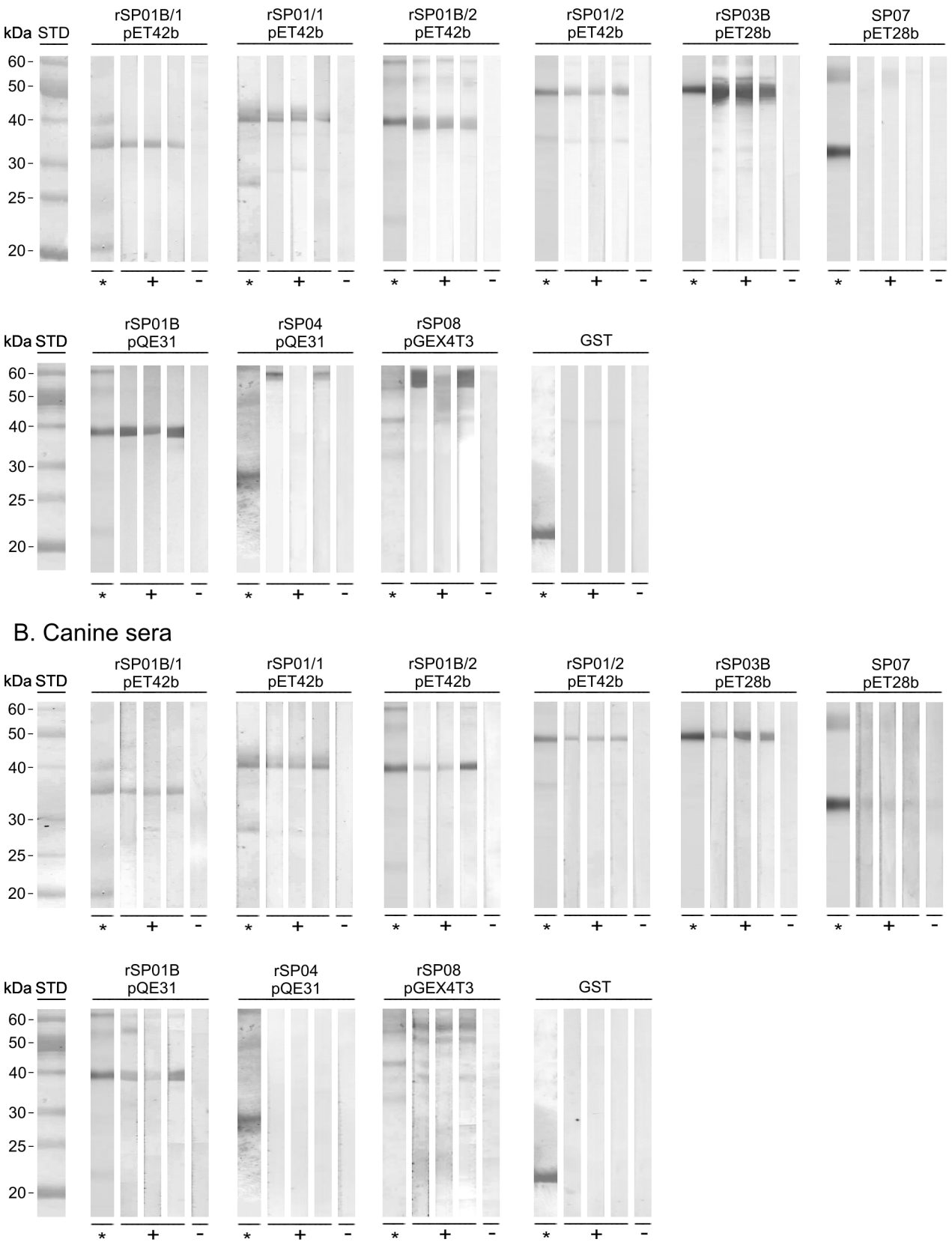

A. Mice sera
kDa STD
rSP01B/1 pET42b
rSP01/1 pET42b
rSP01B/2 pET42b
rSP01/2 pET42b
rSP03B pET28b
SP07 pET28b
60
50
40
30
25
20
* + -
kDa STD
rSP01B pQE31
rSP04 pQE31
rSP08 pGEX4T3
GST
B. Canine sera
kDa STD
rSP01B/1 pET42b
rSP01/1 pET42b
rSP01B/2 pET42b
rSP01/2 pET42b
rSP03B pET28b
SP07 pET28b
kDa STD
rSP01B pQE31
rSP04 pQE31
rSP08 pGEX4T3
GST

Figure 1. Reactivity of recombinant *P. perniciosus* salivary proteins with mice and canine sera using immunoblots. Six recombinant proteins from *P. perniciosus* saliva - yellow protein rSP03B, apyrases rSP01B and rSP01, antigen 5 protein rSP07, ParSP25 protein rSP08 and D7 protein rSP04, expressed in different vectors (pET28b, pET42b, pQE31, pGEX4T3) and a GST tag were tested. Apyrases in pET42b are expressed with either 1 His tag (rSP01/1 and rSP01B/1) or 2 His tags (rSP01/2 and rSP01B/2). Proteins were separated by SDS-PAGE and stained by Coomassie Blue (*) or incubated with mice and canine sera. (**A**) Reaction with sera from three SKH1-hr mice experimentally bitten by *P. perniciosus* females (+) and one non-exposed mouse (−). (**B**) Reaction with sera from three beagles experimentally bitten by *P. perniciosus* (+) and pre-immune serum (−).
doi:10.1371/journal.pntd.0002597.g001

Results

1. Immunoblots with mice and canine sera

All recombinant proteins except antigen 5 protein rSP07 and D7 protein rSP04 were recognized by the sera of all three repeatedly exposed mice; control sera and the GST tag were negative (Fig. 1A). A similar reactivity of recombinant antigens was found with the sera of the three dogs repeatedly exposed to *P. perniciosus* (Fig. 1B): all recombinant proteins except antigen 5 protein rSP07 and D7 protein rSP04 were recognized by the sera of repeatedly exposed dogs. In comparison with mice sera, the reaction of canine sera was less intense for some proteins (yellow protein rSP03B and the apyrases rSP01B/2 and rSP01/2 in pET42b) and fewer nonspecific bands appeared in the immunoblots. Control canine sera were negative (Fig. 1B).

2. ELISA with mice sera

The sera of three bitten and three non-bitten mice were tested by ELISA for the presence of antibodies against the recombinant salivary proteins as well as for the anti-SGH antibodies. Results are summarized in Fig. 2. Bitten mice had a highly elevated antibody response to the following seven recombinant proteins: apyrase rSP01B in all three plasmids, both rSP01 apyrases, yellow protein rSP03B and ParSP25-like protein rSP08. Despite the low number of sera samples tested, five of these seven proteins also showed significant positive correlations with the antibody response to total SGH (rSP01B/1 in pET42b: $r = 0.94$, $p = 0.017$; rSP01B in pQE31: $r = 0.94$, $p = 0.017$; rSP01/1 in pET42b: $r = 0.9$, $p = 0.033$; rSP03B: $r = 0.93$, $p = 0.017$; rSP08: $r = 1.0$, $p = 0.003$). The sixth and seventh proteins - rSP01B/2 and rSP01/2 in pET42b - showed positive correlations but were not significant ($r = 0.77$, $p = 0.103$ for both of them).

3. ELISA with canine sera

The ELISA results of recombinant proteins with eighteen canine sera (covering a wide range of anti-SGH antibody levels) are given in Fig. 3. Highly positive correlations with the reaction against SGH were obtained for the two denatured apyrases rSP01B and rSP01 (for both proteins in pET42b with 1 His tag: $r = 0.91$, $p<0.0001$; for rSP01B with 2 His tags $r = 0.89$, $p<0.0001$; and for rSP01 with 2 His tags $r = 0.91$, $p<0.0001$) and yellow-related protein rSP03B ($r = 0.89$, $p<0.0001$) (Fig. 3). The correlations were not significant for the other four proteins tested (Fig. 3, data not shown for antigen 5 rSP07).

Discussion

In this study, we evaluated the reactivity of six recombinant salivary proteins of *P. perniciosus* with animal sera using immunoblots and ELISA. We chose the sera of mice as model laboratory animals and the sera of dogs as the natural reservoir host of *L. infantum*.

In immunoblots, the recombinant antigens reacted similarly with both mice and canine sera: specific reactions were achieved with both apyrases rSP01B and rSP01 (altogether 5 forms tested), yellow protein rSP03B and ParSP25 protein rSP08. On the other hand, antigen 5 protein rSP07 and D7 protein rSP04 were not recognized by any sera. The only difference between mice and

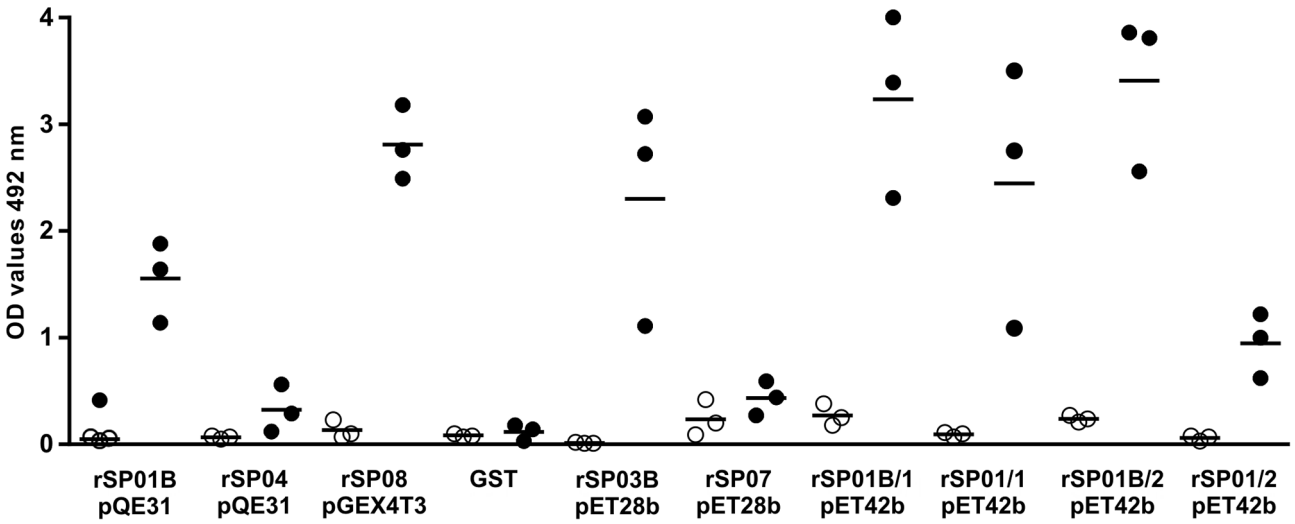
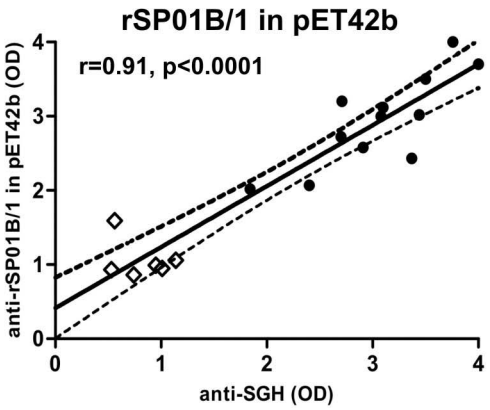
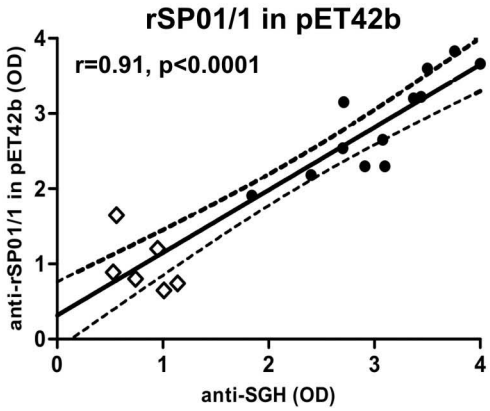
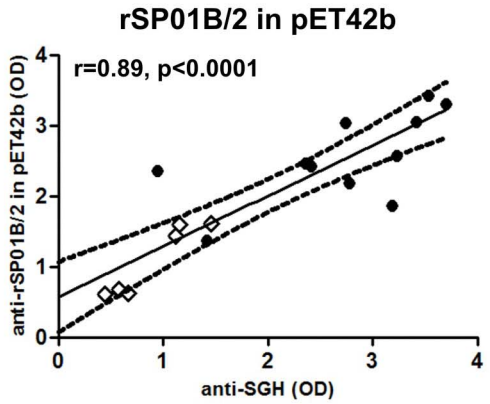
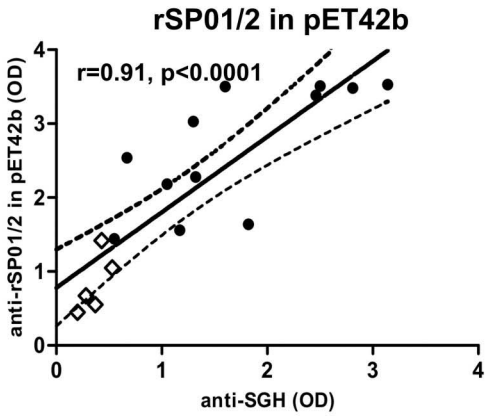


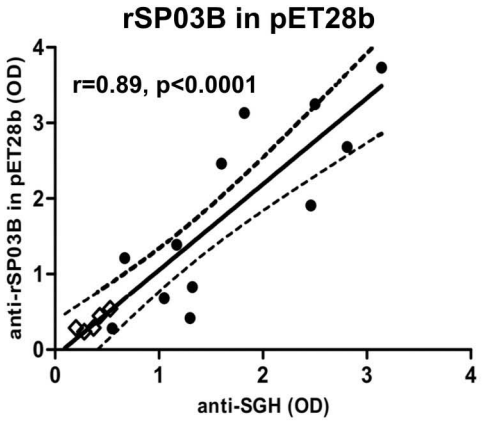
Figure 2. ELISA reactivity of recombinant *P. perniciosus* salivary proteins with mice sera. Six recombinant proteins from *P. perniciosus* saliva - yellow protein rSP03B, apyrases rSP01B and rSP01, antigen 5 protein rSP07, ParSP25 protein rSP08 and D7 protein rSP04, expressed in different vectors (pET28b, pET42b, pQE31, pGEX4T3) and GST tag were tested. Apyrases in pET42b are expressed with either 1 His tag (rSP01/1 and rSP01B/1) or 2 His tags (rSP01/2 and rSP01B/2) Proteins were incubated with sera with sera from three SKH1 mice experimentally bitten by *P. perniciosus* females (black circles). Non-exposed sera of three SKH1-hr mice were used as controls (white circles). Bars show means of optical density values of all three exposed and non-exposed sera. OD = optical density.
doi:10.1371/journal.pntd.0002597.g002

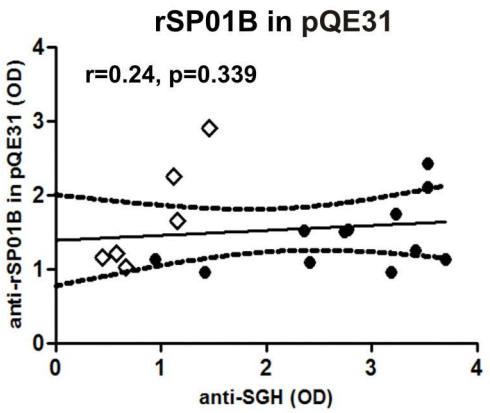

rSP01B/1 in pET42b
r=0.91, p<0.0001
anti-rSP01B/1 in pET42b (OD)
anti-SGH (OD)

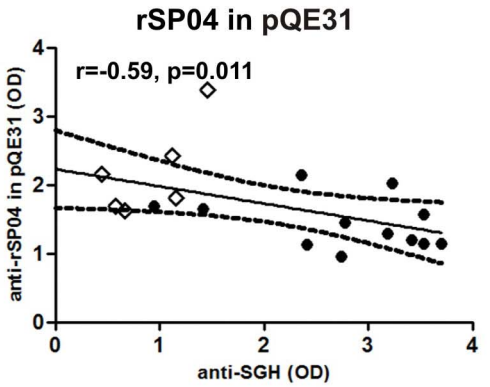

rSP01/1 in pET42b
r=0.91, p<0.0001
anti-rSP01/1 in pET42b (OD)
anti-SGH (OD)


rSP01B/2 in pET42b
r=0.89, p<0.0001
anti-rSP01B/2 in pET42b (OD)
anti-SGH (OD)


rSP01/2 in pET42b
r=0.91, p<0.0001
anti-rSP01/2 in pET42b (OD)
anti-SGH (OD)


rSP03B in pET28b
r=0.89, p<0.0001
anti-rSP03B in pET28b (OD)
anti-SGH (OD)


rSP01B in pQE31
r=0.24, p=0.339
anti-rSP01B in pQE31 (OD)
anti-SGH (OD)


rSP04 in pQE31
r=-0.59, p=0.011
anti-rSP04 in pQE31 (OD)
anti-SGH (OD)

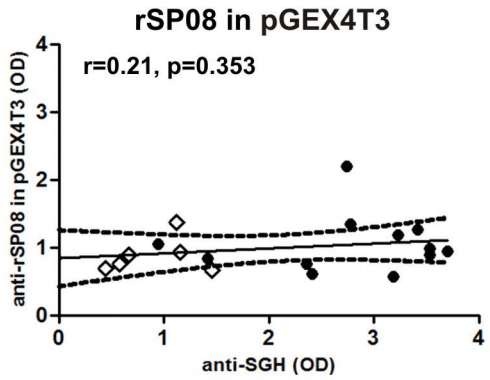

rSP08 in pGEX4T3
r=0.21, p=0.353
anti-rSP08 in pGEX4T3 (OD)
anti-SGH (OD)

Figure 3. ELISA reactivity of recombinant *P. perniciosus* salivary proteins with canine sera. Six recombinant proteins from *P. perniciosus* saliva - yellow protein rSP03B, apyrases rSP01B and rSP01, antigen 5 protein rSP07, ParSP25 protein rSP08 and D7 protein rSP04, expressed in different vectors (pET28b, pET42b, pQE31, pGEX4T3) and GST tag were tested. Apyrases in pET42b are expressed with either 1 His tag (rSP01/1 and rSP01B/1) or 2 His tags (rSP01/2 and rSP01B/2). Proteins were incubated with sera from twelve beagles experimentally bitten by *P. perniciosus* females (black circles). Pre-immune sera of six beagles were used as the controls (white circles). Correlations between the levels of anti-SGH IgG and the levels of anti-recombinant proteins IgG were performed using a Spearman non-parametric test. OD = optical density, r = correlation index, p = p-value.
doi:10.1371/journal.pntd.0002597.g003

canine sera was in the intensity of the positive bands – reactions with mice sera were stronger.

In ELISA, the reactivity of mice and canine sera differed in some aspects; the antibody response to refolded apyrase rSP01B, D7 protein rSP04 and ParSP25 protein rSP08 correlated with anti-SGH response only in mice sera. On the other hand, three denatured recombinant proteins, yellow protein rSP03B and two apyrases, rSP01B and rSP01, correlated significantly with the anti-SGH antibody response using both mice and canine sera. Variations in antigen conformation (denatured vs. refolded) may have led to the exposure of different epitopes. The discrepancy between results found for the refolded rSP01B and rSP08 using ELISA vs. immunoblot could be explained by differences in the exposure of antigens in these techniques; a similar lack of concordance has already been observed between ELISA and immunoblots with mice antibodies against *Phlebotomus sergenti* saliva [17].

Yellow-related proteins were found in the saliva of all sand fly species studied [18,19,20,21]. They were shown to have hemagglutination and lectin-like properties [22]. They also act as high affinity binders of proinflammatory biogenic amines such as serotonin, catecholamines and histamine, suggesting that these proteins may reduce inflammation during sand fly blood-feeding [23]. In *L. longipalpis*, yellow-related protein LJM11 has been proven to have immunogenic properties leading to protective cellular immunity in C57BL/6 mice against leishmaniasis caused by *L. major* [23,24]. Recombinant yellow-related proteins from *P. papatasi* and *L. longipalpis* reacted with the sera of hosts bitten by these sand flies [9,10,11]. Similarly, we have shown here that anti-*P. perniciosus* antibodies also strongly recognize recombinant yellow-related protein from *P. perniciosus* (Figures 1–3). Thus, yellow-related proteins appear to be, in general, promising markers of sand fly exposure.

Apyrases are nucleoside triphosphate-diphosphohydrolases ubiquitously present in the saliva of blood-sucking arthropods. They hydrolyze ADP and ATP in a Ca^{2+}-dependent manner and inhibit ADP-induced platelet aggregation and inflammation to facilitate the blood feeding [18]. In sand fly host models, mouse and hamster antibodies elicited by *P. duboscqi* or *P. perniciosus* saliva recognized bacterially expressed apyrases of *P. duboscqi* and *P. perniciosus*, respectively [25,26].

The three recombinant salivary proteins from *P. perniciosus* are primarily designed for measuring the canine exposure to bites of this sand fly in endemic areas of visceral leishmaniases, and for estimating the risk of *L. infantum* transmission to dogs. Seven sand fly species belonging to the subgenus *Larroussius* are proven or probable vectors of *L. infantum* in the Mediterranean area, with five of them being the most important: *P. perniciosus*, *P. ariasi*, *P. perfiliewi*, *P. neglectus* and *P. tobbi* [5,27]. Among them, *P. perniciosus* is the most abundant in the Western Mediterranean at lower altitudes - in Italy, France, Spain and Portugal. In some of these localities, *P. perniciosus* occurs sympatrically with other *Larroussius* vectors, namely *P. ariasi*, *P. perfiliewi* and *P. neglectus* [27,28,29,30,31]. Studies on the cross-reactivity of anti-*P. perniciosus* antibodies with the saliva of these sand fly species are hampered by difficulties in the maintenance of *Larroussius* colonies; however, based on studies with other sand flies [16,17,32], a certain level of cross-reactivity can be expected only in closely-related species. In this case, such cross-reactivity might be an advantage as all mentioned *Larroussius* species are known to be *L. infantum* vectors [5,27]. It is also important to point out that the specificity of the protein against sympatric sand fly species needs to be studied. Demonstrating sand fly exposure could be pivotal in the discrimination between vector-borne and direct (e.g. congenital, sexual) infections, the latter being hypothesized more and more to justify unexpected autochthonous canine leishmaniasis [33].

In conclusion, we have demonstrated that three denatured recombinant proteins from *P. perniciosus* saliva, the apyrases rSP01B and rSP01 and yellow protein rSP03B, are novel recombinant antigens with great promise in screening canine exposure to this important *L. infantum* vector and for estimating the risk of canine leishmaniases in the western Mediterranean area.

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Author Contributions

Conceived and designed the experiments: PV MJ RM IR. Performed the experiments: JD IMM PS. Analyzed the data: JD IMM IR PV. Contributed reagents/materials/analysis tools: PV MJ RM. Wrote the paper: JD IMM PV MJ RM IR. Created figures: JD IMM. Created striking still image: JD.

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High levels of anti-*Phlebotomus perniciosus* saliva antibodies in different vertebrate hosts from the re-emerging leishmaniosis focus in Madrid, Spain

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ABSTRACT

From July 2009 to date, a leishmaniosis outbreak has occurred in the south-west of the Madrid region (Spain) and has already accounted for more than 450 human cases in an area that comprises a population of approximately 500,000. The causative agent is *Leishmania infantum* and the main vector in the area is *Phlebotomus perniciosus*. Although canine leishmaniosis prevalence in the focus is not higher than the average in the Madrid region, a wild reservoir – the hare – has been implicated. In this study, we examined the exposure of *Leishmania* reservoirs in the area: dogs, hares, and wild rabbits to sand fly bites using the detection of specific IgG antibodies against *P. perniciosus* salivary gland homogenate or recombinant salivary proteins.

Hares collected in a green park adjacent to the focus ($n = 59$) showed positive exposure to *P. perniciosus* bites in comparison to hares from a non-endemic area (Czech Republic, $n = 18$). A significant positive correlation was found between IgG response to yellow protein rSP03B and salivary gland homogenate ($r = 0.902$) and between apyrase rSP01B and salivary gland homogenate ($r = 0.710$). Wild rabbits captured in the study area ($n = 21$) presented higher anti-saliva antibody levels than negative control sera and their IgG response against recombinant salivary proteins were positively correlated with salivary gland homogenate (rSP03B: $r = 0.710$; rSP01B: $r = 0.666$). All sera of dogs from the focus ($n = 34$) showed higher anti-saliva IgG levels than that of non-exposed dogs. Moreover, dogs protected against sand fly bites through the use of topical insecticides and sleeping indoors showed significantly lower antibody levels than the non-protected ones. Antibody response to all three recombinant salivary proteins tested showed positive correlation with salivary gland extract (rSP03B: $r = 0.858$; rSP01: $r = 0.864$; and rSP01B: $r = 0.861$). Data confirmed the exposure of hares, rabbits and dogs to *P. perniciosus* bites in the context of an outbreak of human leishmaniosis in Spain, highlighting their involvement in *Leishmania* transmission by supporting their role as potential reservoirs.

This novel methodology represents a promising tool for further epidemiological studies that would help to design better strategies for the control of leishmaniosis in this area and other foci.

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1. Introduction

Human leishmaniosis is an endemic disease in southern Europe, where the parasite – *Leishmania infantum* – acts as the causative agent while several species of phlebotomine sand flies belonging to the genus *Phlebotomus* serve as vectors. It is considered a zoonotic disease, the dog being the main reservoir (WHO, 2010). In Western Mediterranean countries, sand fly activity is seasonal and the winter corresponds to a sand fly free period. The incidence of visceral leishmaniosis in Europe was recently revised and established between 0.02 and 0.49/100,000 inhabitants per year (Alvar et al., 2012). However, cutaneous leishmaniosis is not frequently notified (Antoniou et al., 2013). Currently, Europe is facing demographic, environmental, climatic and urbanization modifications that are leading to a change in the disease distribution. Expansion of leishmaniosis cases to non-endemic areas in Italy and France has also been documented (Maroli et al., 2008; Dereure et al., 2009; Gramiccia et al., 2013). The emergence of human leishmaniosis foci in Crete, Cyprus and Spain provides clear examples of the dynamic nature of leishmaniosis in Europe (Antoniou et al., 2013).

In the recently described focus in the south-western area of the Madrid region (Spain), 446 cases were reported from July 2009 to December 2012 (6 in 2009, 97 in 2010, 196 in 2011 and 147 in 2012) affecting four municipalities (Fuenlabrada, Leganés, Getafe and Humanes de Madrid) (Arce et al., 2013). Between 12 and 25 cases were notified annually in Madrid for the period of 2000–2009 leading to an increased incidence in the Fuenlabrada municipality from 2.44/100,000 inhabitants in 2009 to 54.2/100,000 inhabitants in 2013 (Antoniou et al., 2013). As expected, *L. infantum* is the parasite species involved in the leishmaniosis focus (Chicharro et al., 2013). Entomological research has determined that the vector is *Phlebotomus perniciosus*, the predominant species in the area (66.1%) where high densities (143.8 sand flies/m^2 in 2012) were found (Arce et al., 2013). Moreover, molecular detection of *L. infantum* in wild-caught *P. perniciosus* ($n = 135$) showed a high prevalence of infection (58.5%), providing evidence of the high transmission events that are taking place in the focus (Jiménez et al., 2013).

The data derived from the analysis of 2070 dogs studied in 2011 and 2012 revealed a low canine seroprevalence of 1.64% in the affected municipalities (Vilas et al., 2012). Therefore, wider studies focusing on potential wild reservoirs resulted in *Leishmania* DNA detection in the spleen and/or skin as well as anti-*Leishmania* antibodies in hares, rabbits and cats (Vilas et al., 2012; Moreno et al., 2014). In addition, xenodiagnosis studies have, for the first time, demonstrated that apparently healthy Iberian hares (*Lepus granatensis*), seropositive to *L. infantum*, were able to infect *P. perniciosus* (Molina et al., 2012). Recent observations on *P. perniciosus* blood meal preferences (Jiménez et al., 2013) and the high population of hares present in the newly constructed periurban green park surrounded by the aforementioned municipalities suggest that hares maintain a high sand fly population in the area and play an active role as reservoirs in a sylvatic transmission cycle linked to the urban periphery, independent of the usual domestic cycle (Molina et al., 2012; Jiménez et al., 2013). A recent study based on molecular detection of the parasite in the spleen of hares captured in different areas of Spain showed a high prevalence of infection (43.6%) in *L. granatensis* and *Lepus europaeus* (Ruiz-Fons et al., 2013).

One of the tools that can be implemented for a leishmaniosis outbreak follow up is detecting the exposure of hosts to leishmaniosis vectors. In this aspect, sand fly salivary proteins were introduced as markers of exposure to sand flies (Barral et al., 2000; Rohoušová et al., 2005; Rohoušová and Volf, 2006; Gomes and Oliveira, 2012). Hosts bitten by sand flies developed specific anti-saliva antibody responses that correlated with the number of bites received (Vinhas et al., 2007; Hostomská et al., 2008; Vlková et al., 2011, 2012). Therefore, host exposure to sand flies can be tracked by evaluating humoral responses against salivary antigens and is being applied using salivary gland homogenate (SGH) (Barral et al., 2000; Clements et al., 2010; Souza et al., 2010; Teixeira et al., 2010; Gidwani et al., 2011) or recombinant salivary proteins (rSP) (Barral et al., 2000; Marzouki et al., 2012; Souza et al., 2010; Vlková et al., 2012). This epidemiological tool has already been used to assess the efficacy of vector control campaigns in endemic areas (Gidwani et al., 2011). Moreover, searching for antibodies against the saliva of blood-feeding insects seems to be a novel approach for the identification of new reservoirs. For instance, detectable levels of antibodies against the saliva of *Lutzomyia longipalpis* present in sera of wild foxes (*Cerdocyon thous*) together with parasite detection in these animals suggested the presence of a sylvatic transmission cycle of *L. infantum* in Brazil (Gomes et al., 2007).

Therefore, the main objective of this study was to analyze the exposure to sand fly bites of the *Leishmania* reservoirs in the focus of Madrid: dogs, hares and a potential reservoir the wild rabbit through the detection of antibodies against *P. perniciosus* SGH and rSP in sera of these animals.

2. Material and methods

2.1. Salivary gland collection from sand flies

P. perniciosus sand flies were maintained at 27 °C and 17:7 h light:darkness photo-period in the Medical Entomology Unit of the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. Sand flies from a colony established from specimens captured in Madrid (Molina, 1991) were used. Salivary glands were dissected from 3 to 5 day-old adult female flies and stored at −70 °C in 20 μl of Tris–NaCl buffer (20 mM Tris, 150 mM NaCl, pH 7.4) in groups of 20 salivary glands until used. SGH was obtained by disrupting the glands through three repeated freeze/thaw cycles.

2.2. Recombinant salivary proteins

Recombinant salivary proteins from *P. perniciosus*, two apyrases, rSP01 and rSP01B (35.5 kDa, GenBank ID: KF257365 and 35.3 kDa, GenBank ID: KF257364, respectively) and a yellow protein rSP03B (43 kDa, GenBank ID: KF257369) obtained from the Laboratory of Vector Biology (Charles University in Prague) were used in denatured

forms (Drahota et al., 2014). In addition, the apyrase rSP01B (35.5 kDa, GenBank ID: KF178455) in its refolded form was produced at the Medical Entomology Unit, ISCIII, (Martín-Martín et al., 2013).

Proteins in their denatured form (both apyrases and the yellow protein) were used for reaction with canine sera as they had been previously assayed as markers of canine exposure to *P. perniciosus* bites (Drahota et al., 2014). On the other hand, the reactivity of the yellow protein in its denatured form and the apyrase in a refolded form (rSP01B) was tested against sera of wild hares and rabbits.

2.3. Sera of wild and domestic animals

2.3.1. Hares and rabbits

Sera were collected from wild hares ($n = 59$) and rabbits ($n = 21$) captured in the green park adjacent to the Fuenlabrada and Leganés municipalities (south-western Madrid region). All hares and 13 rabbits were captured during the winter of 2011/2012. Sera from an additional 8 rabbits captured in March 2013 were also used. Sera of hares from the Czech Republic ($n = 18$) and rabbits maintained at the animal facilities of ISCIII ($n = 11$) served as negative controls. Serum from a rabbit experimentally exposed to *P. perniciosus* bites was used as a positive control.

2.3.2. Dogs

Sera of healthy dogs ($n = 34$) living in the leishmaniosis focus area were also studied. Blood samples were collected during the annual anti-rabies vaccination campaign carried out from May to July 2012 in the Fuenlabrada and Leganés municipalities. Simultaneously, *Leishmania* diagnosis was performed as a part of a *Leishmania* screening campaign on domestic dogs. All dogs included in the study were seronegative for *Leishmania*, as canine leishmaniosis prevalence was too low (Vilas et al., 2012). Therefore, we did not intend to include a relationship between the anti-sand fly saliva antibody levels and *Leishmania* infection.

Serodiagnosis was conducted by detecting specific antibodies against *L. infantum* either by rK39 immunochromatographic test (Kalazar Detect Canine Rapid Test; Inbios) performed by the veterinarian immediately after blood collection, or by using the indirect immunofluorescence antibody test (IFAT) at the WHO Collaborating Centre for Leishmaniosis (ISCIII) according to standard procedures (Alvar et al., 2004). The cut off value indicating a positive result was set at 1:80, which is the internationally accepted IFAT limit (Franco et al., 2011).

Based on the epidemiological information acquired during the blood sample collection, the dogs were classified into two groups according to their potential level of exposure to sand fly bites, as outlined in Table 1. Therefore, young and asymptomatic dogs were included in the low exposure group when meeting certain criteria such as sleeping indoors, treatment with topical insecticides and a history of no travel to other leishmaniosis endemic areas. Additionally, the lack of relation between these dogs and human or canine leishmaniosis cases was established as the owners and other dogs residing in the same household were not leishmaniosis cases. On the other hand, dogs that were not treated with topical insecticides and/or slept outdoors were regarded as the high exposure group. Moreover, sera of 6 non-exposed dogs to *P. perniciosus* bites were used as negative controls (Vlková et al., 2011).

Table 1
Classification of dogs according to their potential exposure to sand fly bites.

Level of exposure of dogs to sand fly bites	Number of dogs	Criteria
Low	17	Age < 2 years; asymptomatic; treatment with insecticides; sleeping indoors; negative serology to *Leishmania* (immunochromatography rK39 and IFAT); no history of travel; no relation with human or canine leishmaniosis cases.
High	17	Asymptomatic; either lack of treatment with insecticides and/or sleeping outdoors; negative serology to *Leishmania* (immunochromatography rK39 and IFAT).

All animals used in the study were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals and approved by ethical committees for animal care and experimentation.

2.4. ELISA

Specific anti-saliva IgG antibodies were measured by ELISA, according to Rohoušová et al. (2005) with minor modifications. Briefly, microtiter plates (Maxisorp, Nunc) were coated either with SGH (0.5 salivary gland per well) or rSP in carbonate buffer ($NaHCO_3$ 28 mM, Na_2CO_3 15 mM; pH 9.5) overnight at 4 °C. Recombinant salivary proteins were used as follows: denatured apyrases rSP01 and rSP01B (6 μg/ml and 9 μg/ml, respectively); denatured yellow protein rSP03B (3 μg/ml) and refolded apyrase rSP01B (35 μg/ml). The plates were blocked with 6% skimmed milk in PBS-Tw at 37 °C for 1 h, washed twice with PBS-Tween (PBS-Tw) and sera diluted in PBS-Tw (1:50) was incubated at 37 °C during 3 h. After repeated washings, goat anti-rabbit IgG (1:2.500, Southern Biotech), or goat anti-dog IgG (1:1.000, Bethyl Laboratories) peroxidase-conjugated antibodies were incubated for 1 h at 37 °C. Following another washing cycle, the plates were developed with orthophenylendiamine (0.5 mg/ml) in McIlwein phosphate citrate buffer (pH 5.5) in the presence of 0.001% (v/v) of H_2O_2 (30%). The reaction was stopped with 10% H_2SO_4 and absorbance was measured at 492 nm using an ELISA reader (ELx800, BioTek). Each serum was tested in duplicate and the experiment was repeated at least twice. Wells without serum (but coated with SGH) served as negative controls. IgG antibody levels are reported as adjusted OD, calculated for each serum as a mean OD value of the duplicated wells minus the OD value of the control wells.

2.5. Western blot

SGH (20 glands per well) or rSP (4 and 9 μg per well for rSP03B and rSP01B, respectively, were separated by SDS PAGE on 12% polyacrylamide gels under denaturing conditions using the Mini-PROTEAN apparatus (Bio-Rad). Proteins were quantified by the Lowry method (Lowry et al., 1951). Protein bands were either stained by Coomassie brilliant Blue (Bio-Rad) for protein visualization or transferred to Amersham Hybond™-P membranes (GE Healthcare). Membranes were then cut into strips (the area corresponding to one well of the 10 well-comb was cut into 5 strips) and blocked at room temperature for 1 h with 5% milk in 0.05% Tris-Tween buffer (Tris-Tw) for Western blot. After washing in Tris-Tw, membranes were incubated for 1 h with diluted sera (1:50). The strips were repeatedly washed and incubated for 1 h with peroxidase conjugated anti-rabbit IgG (1:1.000, Sigma). Immunogenic protein bands were visualized by substrate solution with diaminobenzidine (Sigma) and the reaction was stopped with distilled water.

2.6. Statistical analysis

The non-parametric Mann–Whitney *U* test was performed to statistically compare sets of groups without assuming Gaussian distribution. Spearman *t* test was chosen to establish correlations between two data sets. Both statistical analyses were done using Prism version 5 (GraphPad Software). Statistical significance was considered when the *p*-value was <0.05.

3. Results

3.1. Exposure of wild animals to sand fly bites

ELISA of all hare sera from the focus displayed significantly higher IgG anti-saliva antibody levels than negative controls from the non-endemic area ($p < 0.0001$). All values were greater than the cut off set at $OD_{492} = 0.17$, ranging from low ($OD_{492} = 0.42$) to very high positives ($OD_{492} = 3.95$) (Fig. 1).

The majority of rabbit sera ($n = 17$; 81%) showed anti-saliva antibody levels above the cut off ($OD_{492} = 0.20$), established with sera of non-exposed rabbits from the animal facilities ($p < 0.0001$). The absorbance values varied more than in hares. However, the maximum antibody levels were lower (Fig. 1). No difference was found between anti-saliva antibody values from rabbits captured in 2011/2012 versus 2013 ($p = 0.59$) (Fig. 1).

Sera of both wild hares and rabbits recognized several *P. perniciosus* salivary proteins as demonstrated by the Western blot (Fig. 2). Sera of hares mostly recognized the 48, 44, 31 and 27 kDa protein bands while the most antigenic ones for wild rabbits appeared at 48, 44, 37, 31 and 29 kDa. Apart from these five protein bands, the serum of experimentally exposed rabbit recognized additional bands at 41, 35, 33 and 24 kDa.

There was a significant correlation between IgG antibodies against recombinant salivary proteins (rSP03B and rSP01B) and the total anti-SGH antibodies ($p < 0.0001$).

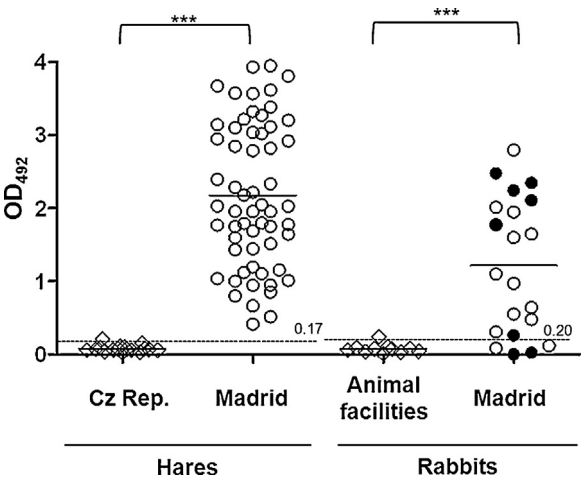

Fig. 1. Anti-*Phlebotomus perniciosus* saliva IgG antibody levels in sera of 59 hares and 21 rabbits. (○) Sera of wild animals collected during winter 2011/12; (●) Sera of wild animals collected in March 2013; (◊) Sera of non-exposed animals. 0.17 OD and 0.20 OD cut off values were established with sera of non-exposed hares and rabbits, respectively. Bars indicate the mean values. Statistically significant differences between groups are marked by *** signs ($p < 0.0001$).

Spearman correlation test results for the rSP03B yellow protein were $r = 0.902$ and $r = 0.710$ for hares and rabbits, respectively, while the rSP01B apyrase showed values of $r = 0.710$ and 0.666, respectively (Fig. 3). In addition, both rSP (35.3 kDa for the apyrase and 43 kDa for the yellow protein) were recognized by sera of most of the wild animals (Fig. 4).

3.2. Exposure of domestic dogs to sand fly bites

Sera from dogs in the leishmaniosis focus area ($n = 34$) showed significantly higher anti-saliva antibody levels than the non-exposed dogs (cut off = 0.24, $p < 0.0001$), (Fig. 5A). Additionally, dogs partially protected against sand fly bites through both the use of topical insecticides and by sleeping indoors showed significantly lower antibody levels than the non-protected ones ($p = 0.021$, Fig. 5B).

The three rSP tested, the apyrases rSP01 and rSP01B, and the yellow protein rSP03B, showed a significant positive correlation with the salivary gland extract ($r = 0.864$, 0.861 and 0.858, respectively; $p < 0.0001$). The lowest data dispersion corresponds to the recombinant yellow protein (Fig. 6).

4. Discussion

This study shows that domestic (dogs) as well as wild animals (hares and rabbits) are exposed to *P. perniciosus* bites in the active leishmaniosis focus in Madrid. Particularly, the results indicate that all hares were exposed to *P. perniciosus*, reinforcing the hypothesis of the existence of a sylvatic cycle independent of the domestic cycle (Molina et al., 2012). Furthermore, the wide range of anti-saliva antibody levels seen in hares and rabbits indicates different ranges of exposure. Anti-rabbit conjugate was used for IgG detection in sera of leporids, as it had also been shown

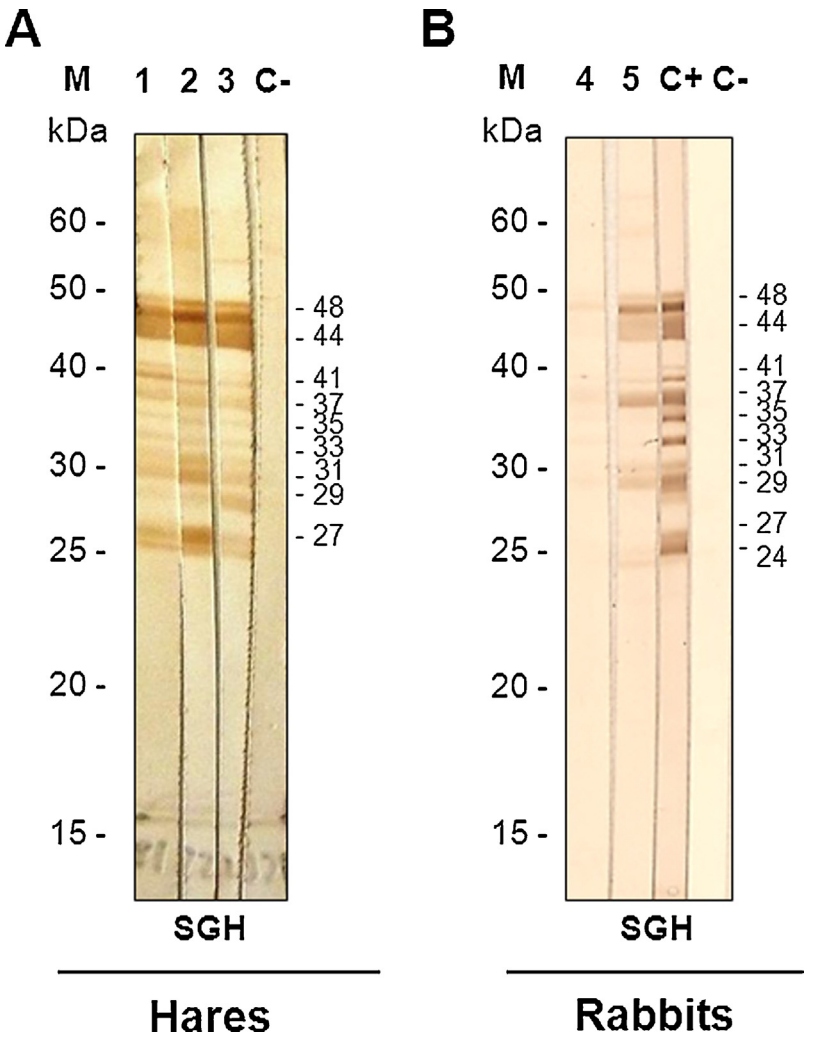

Fig. 2. Panel (A): Western blot of *Phlebotomus perniciosus* SGH with sera of wild hares, designated as 1, 2 and 3. (C−) Negative control serum from a non-exposed hare from the Czech Republic. Panel (B): Western blot of *P. perniciosus* salivary gland homogenate with sera of wild rabbits, indicated as 4 and 5. (C+) Positive serum from a rabbit experimentally bitten by sand flies; (C−) Negative serum from a non-exposed rabbit from the animal facilities. (M) Molecular weight marker Precision Dual Xtra Plus (Bio-Rad).

adequate for IgG detection in hares (Gustafsson et al., 1997; Moreno et al., 2014). Higher anti-saliva levels have been detected in hares over rabbits. However, a higher exposure of hares to sand fly bites cannot be concluded as there might be different affinity/reactivity of rabbit conjugate with hare sera versus rabbit sera or even different antigenicity of salivary proteins for hares and rabbits as observed by Western blot (Fig. 2). Interestingly, hares and rabbits were captured during winter, showing that high anti-saliva antibody levels persisted in sera of wild animals at least from late October, the end of the sand fly season in the Madrid region (Gálvez et al., 2010) until the following winter, when the animals were captured. To date, only a few studies have focused on the kinetics of anti-saliva antibodies (Vinhas et al., 2007; Hostomská et al., 2008; Vlková et al., 2011; Vlková et al., 2012) and to our knowledge, no data regarding the kinetics of specific anti-sand fly antibodies in leporids is available. Therefore, it was not possible to link the high antibody levels observed in sera of hares and rabbits with sand fly salivary antibody kinetics.

In the Iberian Peninsula, infection by *L. infantum* has been described in various species of wild carnivores (Sobrino et al., 2008) and other animals including rodents, horses, sheep and goats (Portús et al., 2002; Fernández-Bellón et al., 2006), suggesting the existence of other independent transmission cycles than just the domestic one. Iberian hares (*L. granatensis*) have been recently identified as active reservoirs in the aforementioned outbreak (Molina et al., 2012) and a high probability of a northward expansion of the Iberian hare was predicted for the future (Acevedo et al., 2012). Also, the European hare (*L. europaeus*) seems to be able to accommodate *L. infantum* infection as well (Ruiz-Fons et al., 2013). Therefore, control measures are essential to reduce this newly described and versatile reservoir and to determine their epidemiological role in other leishmaniosis endemic regions.

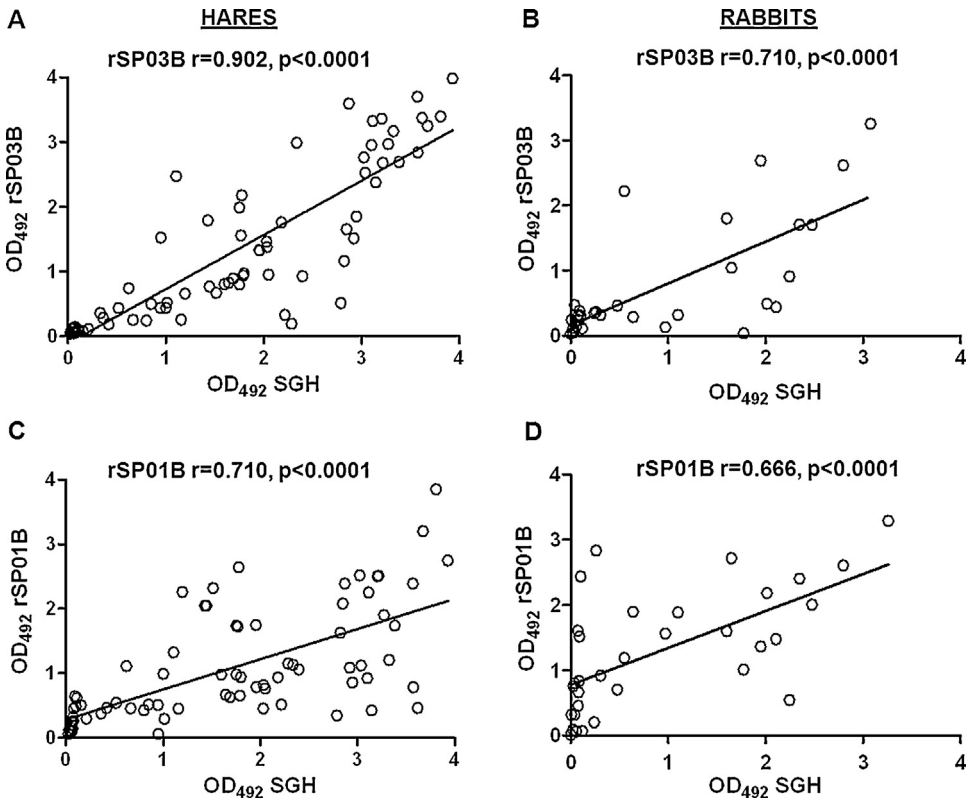

Fig. 3. Correlation between IgG antibodies against SGH and rSP, rSP03B and rSP01B using sera of hares (Panels A and C) or rabbits (Panels B and D).

In rabbits, a single *Leishmania* PCR positive animal (1 out of 162) was reported in southern Spain (Chitimia et al., 2011) and it was hypothesized that they would act as accidental hosts rather than as active reservoirs. Recently, anti-*L. infantum* seroreactivity was detected in 45.7% of rabbits (16 out 35) captured in the focus area of south-western Madrid (Moreno et al., 2014). Although hares and rabbits belong to the same family (Leporidae), they exhibit

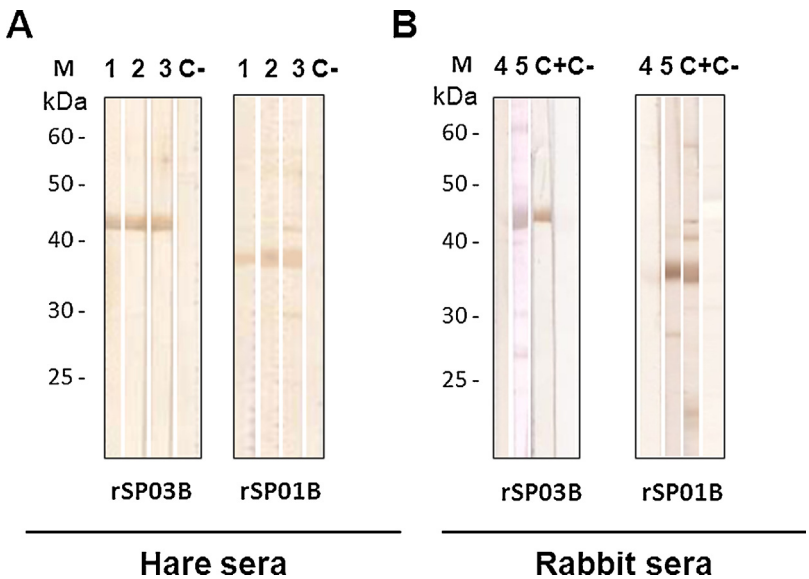

Fig. 4. Panel (A): Western blot of *Phlebotomus perniciosus* rSP - rSP03B and rSP01B- with sera of wild hares, designated as 1, 2 and 3. (C−) Negative control serum from a non-exposed hare from the Czech Republic. Panel (B): Western blot of *P. perniciosus* salivary gland homogenate with sera of wild rabbits, indicated as 4 and 5. (C+) Positive serum from a rabbit experimentally bitten by sand flies; (C−) Negative serum from a non-exposed rabbit from the animal facilities. (M) Molecular weight marker Precision Dual Xtra Plus (Bio-Rad).

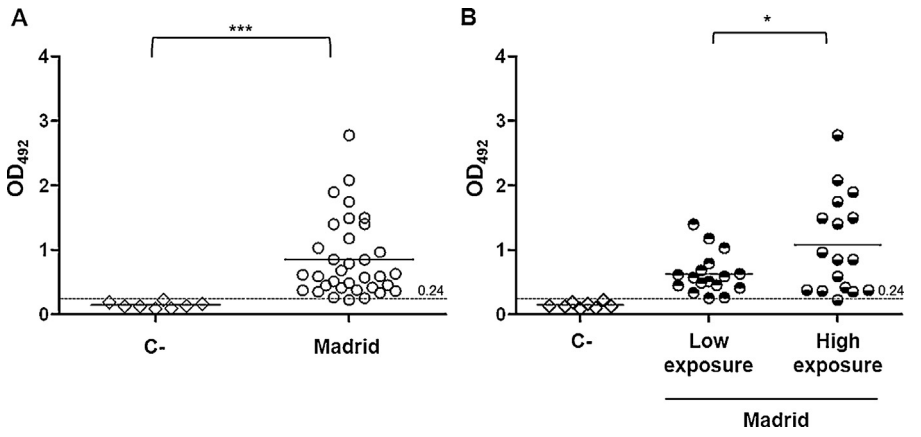

Fig. 5. Anti-*Phlebotomus perniciosus* saliva IgG antibody levels in sera of 34 dogs from Madrid. Panel (A): (○) Sera from dogs living in Madrid ($n = 34$) plotted together; (◊) Sera of non-exposed dogs ($n = 8$). Panel (B): Sera of 34 dogs grouped according to their expected exposure to sand fly bites; (◓) Sera of low exposure group; (◒) Sera of high exposure group; (◊) Sera of non-exposed dogs ($n = 8$). 0.24 cut off value set with sera of non-exposed dogs (◊).Bars indicate the mean values. Statistically significant differences between groups: *** $p < 0.0001$ or * $p < 0.05$, respectively.

remarkable differences in immune responses (Carmo et al., 2006) and may respond to *Leishmania* parasites in a different manner (Moreno et al., 2014). Rabbits are known as highly attractive hosts and a good blood meal source for *Larroussius* species (Killick-Kendrick and Killick-Kendrick, 1991; Benito-De Martín et al., 1994; Volf and Volfova, 2011). Our results indicate that wild rabbits are frequently bitten by *P. perniciosus*, therefore they probably contribute to maintaining high population levels of sand flies in the focus.

Throughout 2012, several control measures were performed in the focus area of south-western Madrid in order to halt the spread of this epidemic (Suárez-Rodríguez et al., 2012). Therefore, differences in reservoir exposure due to a reduced sand fly density would be expected when analyzing animals captured before and after the control measures. However, no differences were found between exposure results of rabbits captured in 2011/2012 versus 2013. Nevertheless, these results may have been hampered by the small numbers of rabbit sera samples available from each year (2011/2012 and 2013). Clearly, more research is needed in order to clarify the role of rabbits in the leishmaniosis epidemiology of this focus.

P. perniciosus salivary antigens immunogenic to hares and rabbits were identified by Western blot. Interestingly, sera from these leporids showed a different pattern of recognition in terms of intensity and immunogenicity. The most antigenic bands appeared at 48, 44, 37, 31 and 27–29 kDa. Based on their coincident molecular weight and on previous experiments regarding salivary antigen proteomic identifications, 48 and 44 kDa bands correspond to yellow proteins (Anderson et al., 2006; Vlková et al., 2011; Martín-Martín et al., 2012) while 35–37 kDa bands may represent apyrases, and 27–29 kDa bands correspond to D7-related proteins and Par25-like proteins. These proteins have been previously identified as antigens in the saliva of *P. perniciosus* using sera of naturally or experimentally bitten hosts (Rohoušová and Volf, 2006; Vlková et al., 2011; Martín-Martín et al., 2012).

Results obtained from dogs living in the focus area showed that these animals were exposed to sand fly bites as their level of anti-saliva antibodies was higher than the cut off established with sera of the non-exposed dogs. Although anti-saliva antibodies can persist until the following sand fly season (Hostomská et al., 2008), it can be assumed that these values correspond at least partially to recent exposure, as canine sera were collected within the sand fly season. As expected, dogs belonging to the group of low potential exposure to sand fly bites showed lower anti-saliva antibody levels than those that were not treated with topical insecticides and/or slept outdoors. Our results supported previous observations by other authors that insecticide-treated dogs received fewer sand fly bites than non-treated ones but still developed

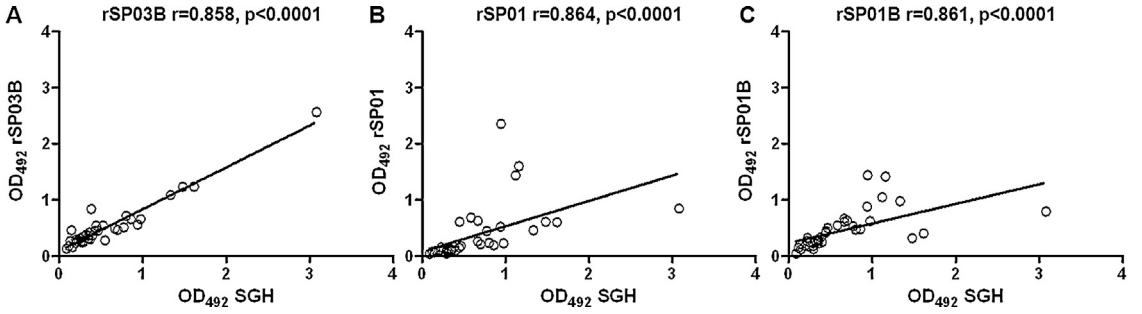

Fig. 6. Correlation between IgG antibodies against SGH and rSP in denatured form rSP03B (Panel (A)), rSP01 (Panel (B)) and rSP01B (Panel (C)) using canine sera.

a low IgG response against sand fly saliva (Hostomská et al., 2008; Vlková et al., 2011). Therefore, these previous observations done with experimentally-bitten dogs were confirmed in our study with naturally-exposed dogs for the first time. Insecticide treatments are widely accepted as the main strategy to control canine reservoir and thus reduce human risk of disease (Alexander and Maroli, 2003). Therefore, monitoring anti-saliva antibodies in canine sera would provide valuable information on control programs efficacy in this focus. Unfortunately, in this study reliable information about anti-saliva response in infected dogs could not be determined, as the canine leishmaniosis prevalence was too low, probably due to the effectiveness of measures taken by owners to protect their dogs against the bite of sand flies in the focus area.

In this study, rSP03B (yellow protein), rSP01 and rSP01B (apyrases) were used in different forms. Proteins in the denatured form were chosen for reaction with canine sera as they had been previously selected as the best markers of canine exposure to *P. perniciosus* bites (Drahota et al., 2014). For wild animals, denatured rSP03B and refolded rSP01B were used. In wild leporids, antibodies against both denatured yellow protein rSP03B and refolded apyrase rSP01B showed a positive correlation with anti-SGH antibodies detected in sera of hares and rabbits. The yellow protein showed the best results for both hares and rabbits, as they presented greater correlation scores when values were compared with SGH. In addition, immunogenicity of these two proteins was confirmed by Western blot. These findings support the idea of replacing the use of SGH by rSP. Apyrases are enzymes typically found in the saliva of sand flies and bed bugs and act as antihemostatic factors by hydrolyzing ATP and ADP. Yellow proteins are widespread within the saliva of insects and their name is derived from the yellow phenotype produced by the mutation of the gene involved. Both protein families are immunogenic to several vertebrate hosts (Drahota et al., 2014; Rohoušová et al., 2012). Recombinant yellow proteins have been successfully tested by others as markers of exposure to sand fly bites (Souza et al., 2010; Vlková et al., 2012). On the other hand, the apyrase rSP01B in its refolded form was shown in previous studies to successfully correlate with salivary gland extract in the murine model but not in the canine one (Drahota et al., 2014). In this study, immunogenicity of rSP01B was confirmed in other reservoirs such as hares and rabbits and it is not surprising as differences in salivary protein recognition patterns by sera of different hosts have been previously documented (Rohoušová et al., 2005; Martín-Martín et al., 2012). In canine sera, high correlation values between antibody levels against rSP and anti-saliva antibodies were obtained for the three assayed proteins as previously observed with experimentally bitten dogs (Drahota et al., 2014), thus confirming the use of these rSP for field applications. The assayed proteins showed similar correlation values with anti-saliva antibodies (rSP01: $r = 0.864$, rSP01B: $r = 0.861$ and rSP03B: $r = 0.858$). However, the recombinant yellow protein seems to be the best candidate as marker of canine exposure to sand fly bites since it presents the lowest data dispersion.

In the case of *Leishmania* detection in vertebrate hosts, previous contact with sand flies is mandatory. However, in the absence of *Leishmania* infection, a positive exposure of vertebrate hosts to sand fly bites can still happen. In this way, detecting sand fly saliva humoral responses in different hosts would contribute to a better understanding of the eco-epidemiology of the disease, with special importance in the aforementioned emerging outbreak.

5. Conclusions

Our results confirmed the exposure of dogs, hares, and rabbits to *P. perniciosus* bites through this novel methodology. This is the first report of anti-saliva antibody levels in naturally bitten domestic and wild animals in Spain and can be considered as a valuable starting point for future studies on the efficacy of anti-vector control measures, especially in the context of an outbreak of human leishmaniosis. In addition, the use of rSP as markers of exposure to sand fly bites was successfully confirmed and validated in animals from the field.

As no data on kinetics of anti-saliva antibodies in wild animals are available, studies on this topic would be essential to supplement field work.

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Summary and conclusions

These theses are comprised of the results from three projects that I participated on during my PhD study. The common aim of these projects was to study specific antibody response in host bitten by sand flies. Particularly, we decided to identify and prepare recombinant salivary antigens as markers of exposure to sandfly bites. The results of the projects, together with their integration into the general knowledge, are briefly outlined in the following paragraphs.

First we have analyzed antigenicity of salivary proteins of *P. perniciosus*, the major European vector of *Leishmania infantum* causing visceral leishmaniases in humans and dogs. We studied IgG antibody response of dogs from two different sources – experimentally bitten dogs from laboratories in Germany and naturally bitten dogs that were housed in open-air shelters in Putignano (Bari province, Apulia, Italy), where *P. perniciosus* is the most abundant phlebotomine sand fly species (Tarallo *et al*., 2010). After detection on western blots we have identified the best antigens by mass spectrometry. Canine sera recognized more than eleven *P. perniciosus* antigenic bands by western blot and the most intense reaction was often observed against a 42 kDa band which was identified by the mass spectrometry as a single protein belonging to the yellow-related protein family (DQ150622 – PpeSP03B). Previously, another yellow-related protein was reported as the major antigen recognized by sera of dogs bitten by *L. longipalpis* in the field (Bahia *et al*., 2007) and the recombinant *L. longipalpis* yellow-related proteins (rLJM11 and rLJM17) were successfully used to screen dog sera from Brazil (Teixeira *et al*., 2010). All canine sera tested recognized additional three major antigens of the 38, 33 and 29 kDa; the 38 and 33 kDa proteins are apyrases (DQ192490 – PpeSP01 and DQ192491 – PpeSP01B) and the 29 kDa antigen represents the antigen 5-related protein family (PpeSP07 - DQ153102). Apyrase in *P. papatasi* saliva was previously found as a human antigen (Rohousova *et al*., 2005) and also a 34 kDa apyrase from *L. longipalpis* was antigenic for dogs (Hostomska *et al*., 2008), therefore we have chosen these four antigens for expression candidates, see below. **These results were published in Vlkova *et al*., 2011.**

Within the second project aimed at improvement of serological studies using recombinant salivary antigens (rSPs) we choose the classical laboratory model, *P. papatasi* and laboratory mice. To produce rSPs we have decided to use bacterial expression system as

it is the relatively simple system with quite good yield. We were aware of the possibility of incorrect protein folding but, as we did not measure the enzymatic activity of the proteins, only their antigenicity, we hoped that properties of major of salivary antigens will not be changed in bacterial expression system. We have chosen four proteins from two families – yellow-related proteins (PpSP42 and PpSP44) and D7-related proteins (PpSP28, PpSP30) as these two families were confirmed to be highly antigenic (Rohousova *et al*., 2005; Marzouki *et al*., 2011). We have expressed these proteins after receiving their cDNA sequences in VR2001-TOPO vector in *E. coli* BL21 (DE3) - pET-42b expression system and then tested their antigenicity on western blots with Balb/c mice sera. The most intensive reaction was detected with the D7-related protein PpSP30, although, this protein was not recognized by all mice sera tested (4 out of 5). Three out of five mice sera reacted with the yellow-related recombinant proteins PpSP42 and PpSP44 and very weak reaction was detected with the D7-related protein PpSP28 in two out of five mice sera. **These results were published in Vlkova *et al*., 2012.**

After this success we have decided to apply the same methodology on *P. perniciosus* saliva. We choose four major antigens selected during the previous study (Vlkova et al., 2011): yellow-related protein PpeSP03B (DQ150622), apyrases PpeSP01 (DQ192490) and PpeSP01B (DQ192491), and the antigen 5-related protein PpeSP07 (DQ153102).

All four proteins were expressed in the same expression system as before the *P. papatasi* antigens: in *E. coli* BL21 (DE3) - pET-42b or pET-28b expression system in two versions with different number of His tags with primers based on the cDNA published sequences (Andrade *et al*., 2006). Three more proteins were expressed by our colleagues in Madrid also in bacterial system but refolded after isolation and based on different cDNA library primers (Martin-Martin *et al*., 2012) – apyrase PpeSP01B (HE974345.1), ParSP25-like protein PpeSP08 (HE974347.1) and D7-related protein PpeSP04 (HE980444.1). Altogether, eleven different recombinant proteins or their various forms were available for further tests.

Nine of eleven from these recombinant proteins (one yellow-related protein and one antigen 5-related protein were excluded) were tested then with sera of Balb/c mice and beagle dogs experimentally bitten by *P. perniciosus* – the same sera as published in Vlkova *et al*. (2011) using immunoblots and ELISA. All recombinant proteins except antigen 5-related protein rSP07 and D7-related protein rSP04 were recognized by the sera of all three repeatedly exposed mice and all three dogs repeatedly exposed to *P. perniciosus*. In

comparison with mice sera, the reaction of canine sera was less intense for some proteins (yellow-related protein and the apyrases) and fewer non-specific bands appeared in the immunoblots. In ELISA, sera of six mice (three non-exposed and three exposed to *P. perniciosus* bites) and eighteen canine sera (covering a wide range of anti-SGH antibody levels) were tested. Bitten mice had a highly elevated antibody response to the following seven recombinant proteins: apyrase rSP01B in all three plasmids, both rSP01 apyrases, yellow-related protein rSP03B and ParSP25-like protein rSP08. For canine sera, highly positive correlations with the reaction against SGH were obtained for the two denatured apyrases rSP01B and rSP01 and yellow-related protein rSP03B. The correlations were not significant for the other four proteins tested. **These results were published in Drahota *et al.*, 2014.**

Finally, three most promissing recombinant proteins of *P. perniciosus*: yellow-related protein rSP03B and two apyrases rSP01B and rSP01 were used as tools for screening of anti-*P. perniciosus* antibody levels in animals living in *L. infantum* endemic regions of central Spain. There is a new and very interesting focus of *L. infantum* near Madrid around the city Fuenlabrada where hares and rabbits were shown to serve as reservoirs, for more details see the Introduction, part 1.3. To understand better the epidemiology of the diseases, we examined the exposure to *P. perniciosus* bites of all three existing reservoirs in the area: dogs, hares, and wild rabbits. We compared IgG antibodies against both types of antigens, *P. perniciosus* salivary gland homogenate or the recombinant salivary proteins. Fifty-nine hares were collected in a green park adjacent to the focus and showed positive exposure to *P. perniciosus* bites in comparison to eighteen hares from a non-endemic area - Czech Republic. ELISA of all hare sera from the focus displayed significantly higher IgG anti-saliva antibody levels than negative controls from the non-endemic area and a significant positive correlation was found between IgG response to yellow-related protein rSP03B and SGH and between apyrase rSP01B and SGH. Eighty-one percent of the 21 wild rabbits captured in the study area also achieved higher anti-saliva antibody levels than negative control sera and their IgG response against recombinant salivary proteins were positively correlated with SGH. All sera of dogs from the focus (n=34) also showed higher anti-saliva IgG levels than that of non-exposed dogs. Moreover, dogs protected against sand fly bites through the use of topical insecticides and sleeping indoors showed significantly lower antibody levels than the non-protected ones. Antibody response to all three recombinant salivary proteins tested

showed positive correlation with SGH. **All these data were published by Martin-Martin *et al*. (2014)** and confirmed the exposure of hares, rabbits and dogs to *P. perniciosus* bites in the context of an outbreak of human leishmaniasis in Spain, highlighting their involvement in *Leishmania* transmission by supporting their role as potential reservoirs (Molina *et al*., 2012) and also confirmed the efficiency of 3 recombinant proteins as fully functional markers of exposure to sandfly bites.

In conclusion, we proved that sand fly recombinant salivary proteins are useful tools to detect specific antibodies against saliva of the sand fly vector. We establish this method routinely in our laboratory. We recommend the use of recombinant salivary proteins as markers of exposure to sandfly bites and, for example, for evaluating the effectiveness of anti-vector campaigns.

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